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Export and Regulatory Properties of MalE Hybrid Proteins in *Escherichia coli*

A thesis submitted in partial fulfilment of the requirements of the
Open University for the degree of Doctor of Philosophy
in Microbiology

July 1999

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ABBREVIATIONS

amp	ampicillin
ATP	adenosine triphosphate
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylene diamine tetraacetic acid
EtOH	ethanol
Exo III	exonuclease III
glc	glucose
h	hour(s)
IPTG	isopropyl- β -D-thiogalactopyranoside
kan	kanamycin
LB medium	Luria-Bertani medium
LPS	lipopolysaccharide
MalE	maltose binding protein
min	minute(s)
OD	optical density
Omp	outer membrane protein
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNAse	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TBE	Tris-borate-EDTA
TCA	trichloroacetic acid
TEMED	<i>N-N-N'-N'</i> -tetramethylenediamine

ABSTRACT

The maltose binding protein (MalE) of *Escherichia coli* is a secreted 370 residue polypeptide which serves as the periplasmic receptor for high-affinity membrane transport of maltose and maltodextrins. Cells tolerate synthesis and translocation across the plasma membrane of large quantities of MalE. Similarly, the outer membrane protein OmpA is not toxic when expressed in physiological amounts. Expression of an *ompA-malE* hybrid gene consisting of the first 274 residues of OmpA and the last 251 residues of MalE was, however, toxic, although synthesis of the OmpA part alone was not. To examine the effect of synthesis of the MalE part of the hybrid, the *malE* fragment was cloned into a vector where it was preceded by the gene coding for the OmpA signal peptide and was inducible by isopropylthiogalactoside.

Upon induction, a polypeptide of the expected size was made, but expression of the truncated MalE turned out to be even more toxic than that of the OmpA-MalE hybrid. The MalE fragment was exported to the periplasm and did not interfere with the export of other secreted polypeptides. A search for suppressors of this toxicity was performed. In a chromosomal gene bank of *E. coli*, such a suppressor was found which coded for a 102 N-terminal residue fragment of the 217 residue protein NlpE (new outer membrane lipoprotein). NlpE is known to combat the toxicity of the cytosolic β -galactosidase (LacZ) when artificially exported to the periplasm. This suppression is achieved by activation of the Cpx two-component signal transduction pathway controlling expression of the periplasmic protease DegP. Increased synthesis of DegP caused degradation of LacZ. The same mechanism appears to operate for the degradation of the MalE fragment. In a *degP* background, the NlpE fragment could not suppress toxicity. Mutants (*cpxA*^{*}) exist which, without any signal, produced increased levels of DegP. In a *cpxA*^{*} strain, the MalE fragment was no longer toxic.

The toxicity caused by the MalE fragment can still not entirely be explained. However, several lines of evidence, such as the expression of the periplasmic spheroplast protein Y in cells producing MalE251, suggest that the most likely explanation for the lethality is a defect in the cell wall of induced cells.

1. INTRODUCTION

1.1. Structure of the Bacterial Cell Envelope

Escherichia coli, like other gram-negative bacteria, possesses a multilayered cell envelope consisting of the plasma membrane, the periplasmic space, and the outer membrane (Fig. 1). In the following, only those aspects of this envelope will be discussed which are relevant to the data presented in the Results section.

1.1.1. Plasma Membrane

The plasma membrane (also referred to as the cytoplasmic membrane) is a thin structure internal to the cell wall, enclosing the cytoplasm of the cell. It is composed of approximately 70 % protein; this fraction is considerably higher than in eukaryotic cell membranes. Using 2D-gel electrophoresis, it has been demonstrated that these proteins represent more than one hundred different types of molecules (Ames and Nikaido, 1976;

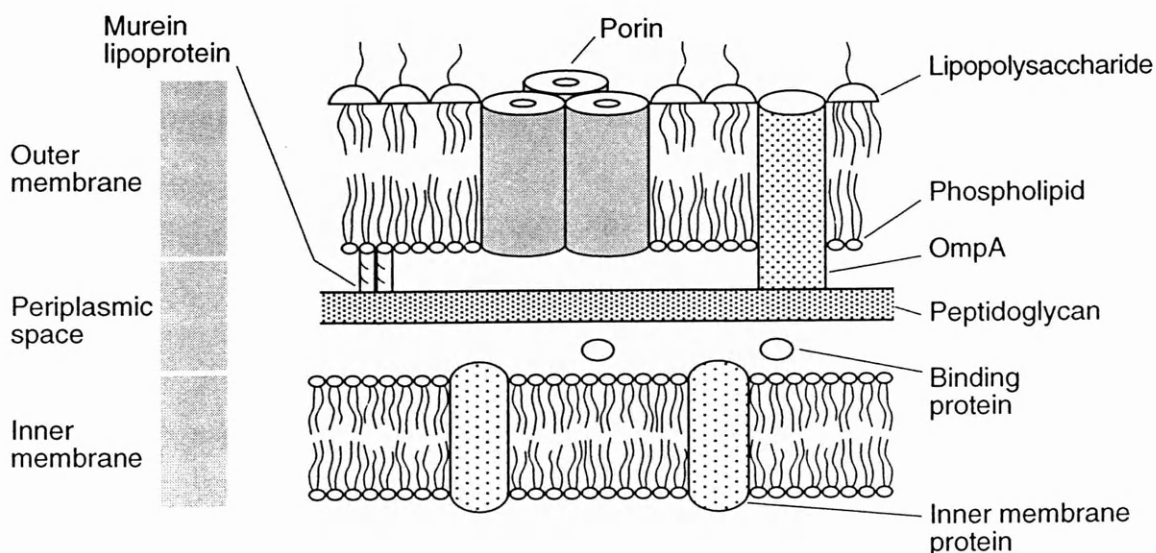


Fig. 1. Schematic model of the cell envelope of *E. coli*. (Modified after Jap and Walian, 1990).

Neidhardt and Umbarger, 1996). The other 30 % of the plasma membrane consist of phospholipids, arranged in two parallel leaflets forming the phospholipid bilayer.

The bacterial plasma membrane serves as a permeability barrier, allowing certain molecules to pass, while preventing the transit of others. The membrane is also the site where a variety of crucial metabolic processes takes place, such as respiration, synthesis of lipids, or import/export of macromolecules. In addition, the membrane contains receptor molecules that help bacteria to sense specifically certain chemicals and to respond to alterations in the environment.

1.1.2. Periplasmic Space

The periplasm (also called periplasmic space) is the region between the inner and the outer membrane. This compartment constitutes about 30 % of the total cell volume of *E. coli* and has a width of approximately 50 nm (van Wielink and Duine, 1990). It has been argued that the periplasm is more a gel than a fluid-filled space. Based on electron microscopy data obtained by the progressive-lowering-of-temperature-embedding technique and by freeze substitution, Hobot and associates (1984) have proposed a model describing the periplasm mainly as a peptidoglycan matrix with large pores. Peptidoglycan or murein represents a polymer of enormous size composed of many components joined covalently together. The polymer contains two carbohydrates, *N*-acetylglucosamine and *N*-acetylmuramic acid, and several amino acids. As a result of being in permanent contact either with proteins of the periplasm or with oligosaccharides, it appears reasonable to assume that proteins in the periplasm are moving much more slowly than in the cytoplasm (Wülfing and Plückthun, 1994). The periplasmic proteins include degradative enzymes like phosphatases, proteases, and nucleases which break down large molecules. Furthermore, detoxifying enzymes, such as the β -lactamases, are secreted into the periplasm of gram-negative bacteria. The

periplasmic space also contains binding proteins for solutes such as sugars, amino acids, and vitamins. One of them, the maltose binding protein (MalE or Mbp) is of special interest to this thesis and will be discussed in more detail below.

1.1.3. Outer Membrane

The outer membrane of gram-negative bacteria is located outside the peptidoglycan layer and has a typical membrane structure. The most abundant outer membrane protein is Braun's lipoprotein (Braun, 1975), a small lipoprotein one third of which is joined covalently to the peptidoglycan and embedded in the outer membrane by its *N*-terminus (Inouye *et al.*, 1972).

Unique constituents of the outer membrane are lipopolysaccharides (LPS). These large amphipathic molecules consist of three parts: the O-antigenic polysaccharide representing the hydrophilic portion, and the core oligosaccharide which is linked to the glycolipidic Lipid A residue. LPS helps to stabilize the structure of the membrane.

One of the most important functions of the outer membrane is to serve as a protective barrier. It prevents or slows down the entry of bile salts, antibiotics, and other toxic substances. On the other hand, the outer membrane is much more permeable than the plasma membrane and permits the passage of small molecules, such as glucose (glc) and other mono- or oligosaccharides. The reason for this phenomenon is the presence of special porin proteins: the osmoporin OmpC, the matrixporin OmpF, the phosphoporin PhoE, and the maltoporin LamB. These porins are extremely resistant to detergents. The OmpF, OmpC, and PhoE porins share a high degree of identity; in the case of OmpF and PhoE, this amounts to 63 % identity (Mizuno *et al.*, 1983).

The 325 residue OmpA protein (Chen *et al.*, 1980) is one of the most abundant polypeptides of the outer membrane of *E. coli*. It is synthesized as a precursor protein with a 21-residue signal peptide. In its native form, OmpA exists as a monomer

(Henning and Koebnik, 1994). The polypeptide consists of a membrane part, ranging from amino acid 1 to 170, and a periplasmic moiety consisting of residues 171 to the C-terminus (Chen *et al.*, 1980; Bremer *et al.*, 1980; Ried *et al.*, 1994). Studies with phage-resistant OmpA mutants have suggested that the membrane part crosses the membrane eight times in antiparallel β -sheet conformation (Morona *et al.*, 1984). Thereby, it most likely forms an amphiphilic β -barrel with relatively long loops protruding into the extracellular space (Vogel and Jähnig, 1986). These loops, together with the LPS, are responsible for the phage receptor activity of OmpA (Morona *et al.*, 1984).

OmpA is a multifunctional polypeptide. In addition to its role as a phage receptor (Datta *et al.*, 1977), it can serve as a mediator in F-factor-dependent conjugation (Schweizer and Henning, 1977; van Alphen *et al.*, 1977). Furthermore, OmpA has been proposed to stabilize the shape of *E. coli* (Sonntag *et al.*, 1978). A pore-forming activity has been reported by Sugawara and Nikaido (1992). However, the rate of penetration of the solutes through the OmpA channel is rather slow and the mechanism is not clear yet.

1.2. Maltose Binding Protein

One of the best characterized constituents of the periplasmic space is MalE. It is encoded by *malE* and serves as the periplasmic receptor for high-affinity membrane transport of maltose and maltodextrins (for review, Ames, 1986). In addition to binding these substrates, MalE interacts with at least three proteins of the cell envelope. Firstly, an interaction with the maltoporin LamB facilitates the diffusion of maltose and maltodextrins through the outer membrane (Wandersman *et al.*, 1979; Heuzenroeder and Reeves, 1980; Boos and Staehelin, 1981; Bavoil and Nikaido, 1981; Bavoil *et al.*, 1983; Neuhaus *et al.*, 1983). Secondly, interaction with the inner membrane methyl-

accepting protein MCP II induces a chemotactic response (Koiwai and Hayashi, 1979; Hayashi and Ohba, 1982; Richarme, 1982). Thirdly, MalE interacts with the permease complex.

The primary structure of MalE and the sequence of its structural gene have been determined by Duplay *et al.* (1984). MalE consists of 370 amino acid residues, corresponding to a molecular mass of 40,661. The protein is synthesized as a precursor with an *N*-terminal 26-residue signal sequence which is cleaved off during or shortly after translocation across the inner membrane (Bedouelle *et al.*, 1980). It is necessary that the precursor exists in an export-competent conformation representing a partially unfolded state. This initial conformation is maintained by binding of the MalE precursor to the chaperone SecB (Weiss *et al.*, 1988; Kumamoto, 1991) and by means of the signal peptide (Park *et al.*, 1988).

1.3. Secretion of Periplasmic and Outer Membrane Proteins

Approximately 20 % of the polypeptides synthesized by gram-negative bacteria are exported to the periplasmic space and the outer membrane (Pugsley, 1993). The proteins located in these two compartments have, during or after synthesis, to be translocated across the plasma membrane. This is achieved in most cases by the use of a signal sequence and the Sec system of proteins. Outer membrane proteins have to overcome an additional problem; they have to cross the periplasmic space to reach the outer membrane. Recent studies suggest that for this translocation step a periplasmic chaperone might be required (Chen and Henning, 1996). In the following, I will present a short description of the factors that might be important for this work.

1.3.1. Signal Peptides

Many secretory proteins including MalE, β -lactamase, and OmpA, possess an

amino-terminal signal sequence (also called leader sequence). Signal sequences are involved in several functions. The signal peptides of the MalE precursor (Park *et al.*, 1988) and that of the β -lactamase precursor (Laminet and Plückthun, 1989) retard the folding of the mature part of the precursor; this is crucial for interaction with the SecB chaperone. Furthermore, the signal sequence is directly recognized by the SecA protein (Cunningham and Wickner, 1989; Fikes and Bassford 1989), the SecY protein (Emr *et al.*, 1981), and the leader peptidase (Dierstein and Wickner, 1985).

1.3.2. Sec Proteins

Seven Sec proteins are known to participate in the general secretion pathway. While it is now certain that SecA, SecY, and SecE are essential for the translocation of general presecretory proteins, the roles of SecB, SecD, SecF, and SecG are less well understood. The SecA protein is unique among the factors that mediate the export in *E. coli*, since it is not only found in the cytosol, but it is also tightly associated with the cytoplasmic membrane (Oliver and Beckwith, 1982; Cabelli *et al.*, 1991). SecA hydrolyses ATP and is an essential component of the protein-translocation ATPase (Lill *et al.*, 1989). The mode of action is likely to be as follows: a particular precursor protein, for example proOmpA, binds to the SecB chaperone (Kumamoto *et al.*, 1989). This precursor-SecB complex then binds to the translocase (Hartl *et al.*, 1990). Translocase consists of the integral membrane protein complex SecY/SecE/SecG (Brundage *et al.*, 1990, 1992; Nishiyama *et al.*, 1993) and a peripheral SecA domain (Oliver, 1993), bound to the membrane by its affinity for SecY/SecE (Hartl *et al.*, 1990) and acidic phospholipids (Hendrick and Wickner, 1991). SecA acts as a receptor for SecB (Hartl *et al.*, 1990) and for the precursor protein where it recognizes both the signal sequence and the mature domain (Lill *et al.*, 1990). The binding of the precursor protein activates the ATPase activity of SecA (Lill *et al.*, 1989).

Genetic studies have suggested that both SecD and SecF are involved in a late step of protein translocation (Gardel *et al.*, 1990; Bieker-Brady and Silhavy, 1992). The two gene products of the *secD* operon, *secD* and *secF*, are the least understood proteins involved in *E. coli* protein export. It can be concluded that, although SecD and SecF are not absolutely required for protein translocation, they are essential for efficient protein export (Pogliano and Beckwith, 1994).

1.3.3. Periplasmic Chaperones

While it has been extensively studied how outer membrane proteins are translocated across the plasma membrane, only little is known about how they finally reach the outer membrane. Two extreme possibilities can be imagined: they may be released in soluble form into the periplasm, or they may use adhesion sites between the two membranes and never enter the periplasm. The existence of such adhesion sites, as first described by Bayer (1968), is still a matter of controversy (Kellenberger, 1990; Bayer, 1991). Arguments favouring a periplasmic route have been brought forward by several authors (Nikaido and Reid, 1990; Sen and Nikaido, 1991; Carlson and Silhavy, 1993; Tommassen and de Cock, 1995; Eppens *et al.*, 1997). In the latter study, Eppens and co-workers demonstrated the existence of periplasmic intermediates of outer membrane proteins during their biogenesis. As a model system, they used the phosphoporin PhoE which, in its wild form, does not contain any cysteine residues. Cysteines were then introduced at positions that are in sufficiently close proximity to induce formation of disulfide bridges. Moreover, these disulfide bridges were positioned at locations within the protein that are not expected to be exposed to the periplasm once the mutant PhoE is correctly assembled into the outer membrane. Using this approach, the authors could show that the periplasmic enzyme DsbA, which catalyses disulfide bridge formation in the periplasm, modulates the folding of the genetically modified

PhoE. It can, therefore, be concluded that the protein must pass through the periplasm on its way to the outer membrane.

If, as suggested by the above studies, the periplasmic route is, indeed, used, assistance in form of a periplasmic chaperone might be required. Recently, it could be demonstrated that a periplasmic protein exists which selectively interacts with outer membrane polypeptides (Chen and Henning, 1996). This protein is the rather basic Skp protein which is located in the periplasm (Thome and Müller, 1991). Inactivation of the chromosomal *skp* gene leads to a drastic reduction in the concentration of several outer membrane proteins, such as OmpA, OmpC, and OmpF. These deficiencies are compensated by the presence of a plasmid carrying the wild-type *skp* gene.

1.4. Goals of the Thesis Project

The 325-residue OmpA protein (Chen *et al.*, 1980) belongs to the very abundant polypeptides of the outer membrane of *E. coli*. It is known from previous investigations (Klose *et al.*, 1988) that the area between residues 154 and 180 of the OmpA protein can direct its periplasmic portion to the outer membrane. The question, therefore, arose whether this area is also able to direct other, normally periplasmic proteins to the outer membrane. For this reason, a gene had been constructed which codes for a tripartite fusion protein consisting of the first 120 residues of MalE, followed by a fragment consisting of residues 154-274 of OmpA, which again is followed by the last 251 amino acid residues of MalE (Klose *et al.*, 1988). A polypeptide of the expected size was synthesized by the cell. However, its synthesis turned out to be lethal. Since the protein consists only of sequences which belong to exported polypeptides, the toxicity was rather unexpected.

The toxicity observed may be due to one (or several) of the following factors:

1. Aberrant folding of the hybrid protein could occur. Thus, translocation across

the plasma membrane may, at least partially, be inhibited, which, in turn, could suppress the translocation of other exported proteins.

2. Periplasmic proteins have to be released from the periplasmic side of the plasma membrane to reach the periplasm. SecD, a component of the Sec protein family (see above), catalyses this release. This late step of protein translocation might be inhibited by the hybrid proteins. If such inhibition occurred, the phenomena could be the same as those caused by conditional *secD* mutants. At the non-permissive temperature of 23°C, severe defects in protein export could be observed. Precursors of various exported proteins accumulated, and cells barely grew at this temperature (Gardel *et al.*, 1987, 1990).

3. The export pathways for periplasmic and outer membrane proteins may diverge at some late step. This late step may be blocked by a hybrid protein, part of which is determined to enter the periplasm, while another part proceeds to the outer membrane. It is likely that the cause of this problem is similar to that observed for inhibition of release (Matsuyama *et al.*, 1993).

To possibly simplify the system, it was first attempted to find out whether synthesis of any one of the hybrid components itself is lethal. An answer to this question might help to reveal the cause of the rather mysterious toxicity.

2. MATERIALS AND METHODS

2.1. Media, Chemicals, and Enzymes

Unless listed below, chemicals were obtained from E. Merck (Darmstadt, Germany).

Acrylamide: Roth, Karlsruhe, Germany

Agarose: BioRad, Richmond, Virginia, USA

Alf-Sequencing-Kit: Pharmacia Biosystems, Freiburg, Germany

Ampicillin: Bayer, Leverkusen, Germany

Amplify: Amersham Buchler, Braunschweig, Germany

Bacto Agar: Difco Laboratories, Detroit, Michigan, USA

Bacto Tryptone: Difco Laboratories, Detroit, Michigan, USA

Bacto Yeast Extract: Difco Laboratories, Detroit, Michigan, USA

Bovine Serum Albumin (BSA): Roth, Karlsruhe, Germany

α -chloronaphthol: Fluka, Buchs, Switzerland

Cloned *Pfu* DNA Polymerase and Buffer: Stratagene, Heidelberg, Germany

Coomassie-Brilliant Blue R250: Serva, Heidelberg, Germany

DNA Ladder: GibcoBRL Life Technologies, Gaithersburg, Maryland, USA

dNTPs: Boehringer Mannheim, Mannheim, Germany

Ethylene Diamine Tetraacetic Acid (EDTA): Serva, Heidelberg, Germany

Ethidium Bromide: Fluka, Buchs, Switzerland

Exonuclease III: Boehringer Mannheim, Mannheim, Germany

Goat-anti-Rabbit IgG Peroxidase Conjugates: Dianova, Hamburg, Hamburg

Isopropyl- β -D-Thiogalactopyranoside (IPTG): Sigma-Aldrich Chemie, Deisenhofen, Germany

Klenow Enzyme: Boehringer Mannheim, Mannheim, Germany

³⁵S-Methionine: DuPont de Nemours, Bad Homburg, Germany

Nitrocellulose Membrane: Schleicher & Schuell, Dassel, Germany

Oligonucleotides: MWG, Biotech, Ebersberg, Germany

Pansorbin cells (*Staphylococcus aureus*): Calbiochem, La Jolla, USA

Plasmid-Midi-Kit including Columns: Qiagen, Hilden, Germany

Rainbow Molecular Marker: Amersham Buchler, Braunschweig, Germany

Restriction Endonucleases: Boehringer Mannheim, Mannheim, Germany

RNAseI: Boehringer Mannheim, Mannheim, Germany

S1-Nuclease: Boehringer Mannheim, Mannheim, Germany

Sodium Dodecyl Sulphate (SDS): Serva, Heidelberg, Germany

T4 DNA-Polymerase: Boehringer Mannheim, Mannheim, Germany

Triton-X-100: Serva, Heidelberg, Germany

Trypsin Inhibitor (from soy bean): Boehringer Mannheim, Mannheim, Germany

Trypsin: Boehringer Mannheim, Mannheim, Germany

Tween20: Serva, Heidelberg, Germany

2.2. Bacterial Strains

The strains of *E. coli* K12 used in the present study are listed in Table 1.

Table 1: Bacterial Strains		
Strains	Genotype	Source
MC4100	F ⁻ , <i>araD139</i> , Δ <i>lacU169</i> , <i>rpsL</i> , <i>relA</i>	Casabadan, 1976
MC4100rF'	Δ (<i>lac-proAB</i>), <i>araD139</i> , <i>rpsL</i> , <i>relA</i> , <i>thi</i> , <i>recA1</i> , (F', <i>traD36</i> , <i>proAB</i> , <i>lacI</i> ^q , <i>lacZ</i> Δ M15)	MacIntyre <i>et al.</i> , 1991
JM109	<i>endA</i> , <i>recA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>hsdR17</i> , (<i>rk</i> ⁺ , <i>mk</i> ⁺), <i>relA1</i> , <i>supE44</i> , Δ (<i>lac-proAB</i>), (F', <i>tra</i> Δ 36, <i>proAB</i> , <i>laqI</i> ^q , <i>lacZ</i> Δ M15)	Yanisch-Perron <i>et al.</i> , 1985
CLC289	MC4100, <i>cpxA17</i>	Danese <i>et al.</i> , 1995

2.3. Plasmids

The plasmids employed for the present investigation are listed in Table 2.

Table 2: Plasmids	
Plasmid	Source
pRD87	Freudl <i>et al.</i> , 1985
pDS5	Stueber <i>et al.</i> , 1984
ppD1	Duplay <i>et al.</i> , 1984
pINompA-2	Ghrayeb <i>et al.</i> , 1984
pK184	Jobling and Holmes, 1990
pOM25	Cole and Raibaud, 1986
pAME	Present study
pME1	Present study
pME1-cat	Present study
pME2	Present study
pK184-Sac	Present study
pK184-Pst	Present study
pK184NE	Present study

2.4. Oligonucleotide Primers

The oligonucleotide primers used are summarized in Table 3.

Table 3: Oligonucleotide Primers		
Primer	Sequence	Purpose
MZ-S1	5'-GCTATCGCGATTGCAGTG-3'	DNA sequencing
MZ-S2	5'-CACATGAATGCAGACACC-3'	DNA sequencing
MZ-S3	5'-TCAAGCGTCAAGTCGACGCGGCAAAGTG-3'	DNA sequencing
MZ-S4	5'-GTAGCGCTGAAGTCTTACGAG-3'	DNA sequencing
MZ-S5R	5'-TTACTTGGTGATACGAGTCTGCGC-3'	DNA sequencing
MZ-S6R	5'-CTGAACGGTCTGCTTATAGG-3'	DNA sequencing
MZ-Ex1	5'-CCGAAAGCTTCAGCATTAGGTCTTGGCA TGCGCAGCGGCCGCGTCTTTCAGGGCTTC-3'	PCR/Exo III
MZ-Ex2	5'-GAGCTGGCGAAAGAGTTCCTC-3'	PCR/Exo III
MZ-Sal	5'-TCAAGCGTGAAGTCGACGCGGCAAAGTG-3'	PCR/ <i>nlpE</i> fragment
Universal primer	5'-GTTTTCCAGTCACAGC-3'	PCR/ <i>nlpE</i> fragment

2.5. Growth Conditions

Cells were grown in Luria-Bertani medium (LB medium; amounts per litre: bacto tryptone, 10 g; bacto yeast extract, 5 g; NaCl, 5 g). To prepare solid medium, 15 g bacto-agar were added. For labelling with [³⁵S]methionine, M 9 minimum medium (amounts per litre: Na₂HPO₄, 6 g; KH₂PO₄, 3 g; NaCl, 0.5 g; NH₄Cl, 1 g; thiamine hydrochloride, 1 g) (Miller, 1972) was used. After autoclaving, 10 ml of a 0.01 M solution of CaCl₂ plus 1 ml of a 1 M solution of MgSO₄ were added. Supplements were glc (0.5%), isopropylthiogalactoside (IPTG, 1 mM), ampicillin (amp; 50 µg/ml), chloramphenicol (30 µg/ml), and kanamycin (30 µg/ml).

2.6. Transformation of Bacterial Cells

2.6.1. Preparation of Competent Cells

2.6.1.1. *CaCl₂-Method* (Cohen *et al.*, 1972). An overnight culture was diluted 1:50. Cells were grown to an OD₆₀₀ of approximately 0.5 and centrifuged at 5,000 rpm for 5 min at room temperature. The pellet was resuspended in 4 ml CaCl₂ (100 mM) and left on ice for 30 min. Following a second centrifugation (5 min, 5,000 rpm, 4°C), cells were resuspended in 0.5 ml ice-cold 100 mM CaCl₂ and left on ice for at least 30 min. 250 µl were taken for each transformation. Using this procedure, the competent cells could be stored for up to three days at 4°C.

2.6.1.2. *PEG-MgCl₂ Method* (Chung *et al.*, 1989). Following a 1:50 dilution of an overnight culture, cells were grown to an OD₆₀₀ of 0.5. They were centrifuged for 5 min at 5,000 rpm at room temperature, placed on ice and resuspended in 0.5 ml ice-cold polyethylene glycol (PEG) solution (10% PEG 6000 plus 50 mM MgCl₂ in LB), to which 10 µl dimethylsulfoxide (DMSO) were added. The cells could be used

immediately for transformation. Their viability decreased after 2 h at 4°C.

2.6.2. Transformation Procedure

Plasmid DNA (100-300 ng) was added to 250 µl of competent cells, and the mixture was incubated on ice for 30 min. The cells were heat-shocked for 2 min in a 42°C water bath and placed on ice for 2 min. 1 ml of LB medium was added, and the cells were shaken either for 30 min at 37°C, or for 45 min at 28°C, to allow expression of the antibiotic resistance gene.

2.7. Preparation of Spheroplasts

E. coli cells containing plasmid pME1 were grown to an OD₆₀₀ of 0.7-1.0 in LB medium. The cells obtained from 10 ml of the culture were harvested by centrifugation and suspended in a solution consisting of 10 mM Tris-HCl (pH 7.5) and 0.75 M sucrose (Matsuyama *et al.*, 1993). After centrifugation at 4,000 rpm for 10 min, the cells were resuspended in the same buffer and then converted to spheroplasts as described by Osborn *et al.* (1972). The final volume of the spheroplast suspension was 2.1 ml. About 90% of the cells were converted to spheroplasts as judged by phase contrast microscopy. 500 µl of the spheroplast suspension were added to 1.25 ml of an LB-sucrose (0.25 M) medium and incubated with IPTG or glc for various times.

2.8. Cell Fractionation

Cells carrying plasmid pME2 were grown in LB medium supplemented with either glc or IPTG. After 45 min, cells were harvested by centrifugation and fractionated by spheroplast preparation (Betton and Hofnung, 1996). The cell pellets,

normalized to the same absorbance reading at 600 nm ($5 \cdot 10^8$ cells/ml), were suspended in 10 mM Tris-HCl (pH 7.5) containing 0.7 M sucrose and 1 mM phenylmethylsulphonyl fluoride. Lysozyme (0.2 mg/ml) and EDTA (10 mM) were added, and the two suspensions were incubated for 20 min at 4°C. The samples were then centrifuged for 5 min at 10,000 rpm in an Eppendorf centrifuge. The supernatants, corresponding to the periplasmic fraction, were carefully removed. The pellets were resuspended, washed, freeze-thawed twice, and centrifuged again for 5 min at 10,000 rpm. The supernatants (cytoplasmic fraction) were removed, and the pellets (membrane fraction) resuspended in Tris-HCl buffer and washed.

2.9. Selection for IPTG-Resistant Mutants

Plasmid pME1-cat was transformed into strain MC4100. Eight single colonies were picked and grown in LB supplemented with amp (100 μ g/ml), chloramphenicol (30 μ g/ml), and glc (0.5%) at 28°C. Cells were plated on LB plates containing amp (100 μ g/ml), chloramphenicol (30 μ g/ml), glc (0.5%), and IPTG (1 mM). Colonies of cells resistant to IPTG at 28°C appeared with frequencies of 10^{-6} - 10^{-7} . Replica plating onto the same medium and incubation at 43°C revealed that approximately 10^{-3} of the IPTG-resistant mutants were also temperature-sensitive, i.e., did not grow at this temperature.

2.10. Protein Techniques

2.10.1. Preparation of Cell Membranes and Whole Cells

For cell membrane preparations, cells were grown to an OD₆₀₀ of 0.8-1.5 and subsequently centrifuged at 4,000 rpm for 10 min. Following centrifugation, the cells

were frozen at -20°C for at least 6 h. They were resuspended in 8 ml H_2O plus 40 μl ethylene diamine tetraacetate (EDTA), transferred into metal beakers, and subjected to sonification in a Branson sonifier on ice two times for 45 s. The suspension was centrifuged for 70 min at 18,000 rpm. The resulting pellet was resuspended in 0.85 % NaCl and centrifuged in an Eppendorf centrifuge at 14,000 rpm for 30 min. Finally, the pellet was resuspended in an appropriate volume of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. For whole cell preparations, cells of a late logarithmic-cell culture were sedimented for 10 min. These cells were resuspended in SDS sample buffer [Tris-HCl (pH 6.8), 0.05M; SDS, 3 %; β -mercaptoethanol, 3 %; glycerol, 10 %; bromophenol blue, 1 %] and sonicated for 5 s.

2.10.2. Electrophoresis of Protein Samples and Immunoblot Analysis (Western Blot)

Membrane preparations and whole cells were subjected to electrophoresis on 12.5 % polyacrylamide gels according to Laemmli *et al.* (1970). Samples were heated to 95°C for 5 min in SDS sample buffer before loading onto the gel. Electrophoresis was carried out at 17 mA overnight. For immunoblot analysis, proteins were transferred to nitrocellulose sheets using an LKB Novablot electrophoresis apparatus. After the transfer, the nitrocellulose sheets were blocked with 3 % bovine serum albumin (BSA) for 1 h at 37°C and subsequently incubated for 1 h with antibody directed against MalE and washed two times for 30 min with phosphate-buffered saline (PBS), pH 7.5, 0.1 % Tween-20, and reacted for 1 h with a goat anti-rabbit IgG peroxidase conjugate. After two washes for 30 min each, proteins were visualized with 60 μg α -chloronaphthol in 20 % ethanol and 150 μl 30 % H_2O_2 .

2.10.3. Preparation of Proteins Produced from Spheroplasts

After induction, the spheroplast culture was centrifuged at 8,000 rpm for 10 min. The pellet was resuspended in sodium dodecyl sulphate (SDS) sample buffer. The supernatant was subjected to trichloroacetic acid (TCA) precipitation (10% TCA for 30 min at 4°C). After centrifugation, the resulting pellet was resuspended in sample buffer and neutralized with ammonia vapour.

2.10.4. Preparation of Proteins Produced by Cell Fractionation

The three fractions (periplasmic, cytoplasmic, and membrane fraction) obtained by cell fractionation were mixed with an equal volume of 2% SDS sample buffer (0.1 ml) and heated to 95°C for 5 min. The samples were analysed by SDS-polyacrylamide gel electrophoresis (see 2.10.2, “Materials and Methods: Electrophoresis of Protein Samples and Immunoblot Analysis”).

2.10.5. Pulse-Chase Experiments and Immunoprecipitation

For pulse-chase experiments, exponentially growing cells were concentrated fivefold, induced with IPTG (1 mM) for 5 min, and labelled at 25°C for 60 s with 5 µl [³⁵S]methionine (50 µCi/ml; DuPont NEN, 43 TBeq/mmol). Following addition of L-methionine (200 µg/ml), samples were taken at indicated times, stored on dry ice, and immunoprecipitated with rabbit anti-MalE antiserum (MacIntyre *et al.*, 1991) or rabbit anti-OmpA antiserum (Henning *et al.*, 1979). For this purpose, 30 µl Tris-SDS buffer [SDS, 12%; Tris-HCl (pH 7.5), 0.3 M] was added to the frozen sample and heated immediately to 95°C for 5 min. After the samples had cooled down, 120 µl of 2 M

NaCl plus the appropriate antibody serum were added to each sample. Following an incubation of 1 h at 37°C, 120 μ l of Pansorbin cells (*Staphylococcus aureus*) were added. After 30 min incubation (37°C), the cells were centrifuged for 5 min at 14,000 rpm in an Eppendorf centrifuge. The pellet was washed two times with 1 ml wash buffer [Tris-HCl (pH 7.5), 10 mM; NaCl, 0.9%; EDTA, 0.1 mM; SDS, 0.02%] and resuspended in 60 μ l of SDS-PAGE sample buffer. For the SDS-PAGE, 30 μ l were used. Following electrophoresis, the proteins were fixed in a solution containing 225 ml methanol, 225 ml H₂O, and 50 ml acetic acid for 30 min. The gel was soaked and agitated for another 30 min in Amplify (Amersham Buchler). The vacuum-dried gel was exposed to X-ray film (Cromex4, DuPont) and exposed at -70°C for 2-7 days.

2.10.6. Trypsin Digestion

An overnight culture of cells was diluted 1:50, and the cells were incubated under three conditions for 1 h each: with IPTG (1 mM), IPTG (1 mM) plus glc (0.5%), and glc (0.5%). The samples were centrifuged, and cells were resuspended with ice-cold Tris-HCl buffer, pH 8.0 (30 mM). To render the cells permeable, an equal volume of an ice-cold solution consisting of 40% sucrose, 20 mM EDTA, and 60 mM Tris, pH 7.8, was added. These cells were incubated with trypsin (0.1 mg/ml for cells carrying plasmid pME1; 0.5 mg/ml for cells carrying plasmid pME2) for 2 h at 4°C. The reaction was terminated with soybean trypsin-inhibitor (0.15 mg/ml for cells carrying plasmid pME1; 0.75 mg/ml for cells carrying plasmid pME2) for 15 min at room temperature. Cells were centrifuged for 30 min at 14,000 rpm in an Eppendorf centrifuge. The resulting pellet was washed twice with 0.85% NaCl and resuspended

in an appropriate volume of SDS-PAGE sample buffer. Trypsin-digested polypeptides were analysed by Western blotting using rabbit antibody directed against MalE (see 2.10.2, “Materials and Methods: Electrophoresis of Protein Samples and Immunoblot Analysis”).

2.11. DNA Techniques

2.11.1. Preparation of Plasmid DNA

2.11.1.1. Mini-Preparation of Plasmid DNA. 3 ml overnight culture were centrifuged in an Eppendorf centrifuge at 12,000 rpm for 5 min. The pellet was resuspended in 100 μ l P1-buffer (100 μ g/ml RNaseA, 50 mM Tris-HCl, 10 mM EDTA pH 8.0). 100 μ l NaOH (200 mM) plus SDS (1%) were added. The tubes were gently shaken and incubated for 10 min at room temperature. After addition of 100 μ l 5 M potassium acetate (pH 6.0), the vials were kept on ice for 15 min, followed by centrifugation at 14,000 rpm for 20 min. The supernatant was transferred into a new tube and an equal amount of phenol/chloroform/isoamyl alcohol (24:24:1, v:v:v) was added. Following extraction with chloroform, the aqueous phase was carefully removed and transferred into new tubes to which twice the volume of 100% ethanol was added. The tubes were shaken and left on ice for 10 min. After centrifugation at 14,000 rpm for 20 min, the DNA pellet was carefully washed with 70% ice-cold ethanol, briefly dried in a speed-vac centrifuge, and resuspended in 100 μ l double-distilled H₂O containing 0.1 mg/ml RNase.

2.11.1.2. Large Scale Preparation of Plasmid DNA. Large scale preparations were carried out by one of the following two methods.

(a) QIAGEN preparation. For the plasmid midi preparations, the QIAGEN-Plasmid-Kit in combination with QIAGEN-Tip-100 columns was used. The isolation was carried out according to the procedure recommended by QIAGEN. Typically, 100 μ g of plasmid DNA were obtained from 30 ml of *E. coli* LB culture for high-copy number plasmids, or from 100 ml LB culture for low-copy number plasmids.

(b) CsCl gradient purification. Cells of an overnight culture (500-1,000 ml) were centrifuged at 10,000 rpm for 15 min. The pellet was resuspended in 8 ml lysis mix (50 mM glc, 10 mM EDTA, 25 mM Tris-HCl, 20 mg lysozyme) and left at room temperature for 5 min. Then, 16 ml 0.2 M NaOH/1 % SDS were added. After 5-10 min incubation, 12 ml 5 M potassium acetate (pH 5) were added and the tubes were inverted several times. Following an additional 30 min incubation on ice, the debris was pelleted at 14,000 rpm for 15 min. The volume of the supernatant was determined, and twice the volume of ethanol (100%) was added. An incubation period of 10 min at room temperature followed. After centrifugation (12,000 rpm, 15 min), the pellet was dried in a vacuum dryer and resuspended in 14 ml TE buffer, pH 7.5. Subsequent to the addition of 0.95 g CsCl/ml TE plus 10 mg ethidium bromide/ml TE, the cells were centrifuged at 40,000 rpm for 10 h. The ethidium bromide band containing the plasmid DNA was isolated with a syringe. Ethidium bromide was removed by two extractions with isoamyl alcohol. CsCl was removed by dialysis against TE buffer. The plasmid DNA was finally precipitated with ethanol.

2.11.2. Polymerase Chain Reaction (PCR) (Saiki et al., 1985)

The PCR was carried out in a Perkin Elmer Thermocycler. The typical reaction mixture is summarized in Table 4.

Table 4: PCR Mix

Component	Volume	Concentration
<i>Pfu</i> DNA polymerase	0.5 μ l	2.5 U
10 \times Reaction buffer	10 μ l	1 \times
dNTPs (1.25 mM)	16 μ l	0.2 mM
Primer 1	1 μ l	1 μ M
Primer 2	1 μ l	1 μ M
Template DNA	1 μ l	10 ng
Sterile distilled H ₂ O	ad 100 μ l	

The composition of the reaction buffer is shown in Table 5.

Table 5: Cloned *Pfu* DNA Polymerase 10 \times Reaction Buffer

Component	Concentration
Tris-HCl (pH 8.8)	200 mM
KCl	100 mM
(NH ₄) ₂ SO ₄	100 mM
MgSO ₄	20 mM
Triton X-100	1 %
Nuclease-free bovine serum albumin	1 mg/ml

Pfu DNA polymerase was employed, since the enzyme possesses a 5'→3' exonuclease activity which ensures a reduction of mistakes during synthesis by a factor of 100 in comparison to *Taq*-Polymerase. The cycle conditions were as follows: 5 min 95°C, 1 min denaturation at 94°C, 1 min annealing at 55°C, and 3 min synthesis at 72°C. A total of 30 cycles were run with 6 s extension time of the synthesis period per cycle.

2.11.3. Isolation of DNA Fragments

Following agarose gel electrophoresis, the agarose slice containing the DNA band of interest was frozen either on dry ice for 1 h, or at -20°C overnight. The frozen agarose slice was centrifuged at 14,000 rpm for 30 min, and the supernatant was collected. The pellet was resuspended with 20 µl TE buffer and centrifuged at 14,000 rpm for 30 min. The DNA was collected by ethanol precipitation.

2.11.4. Exonuclease III Deletions

2.11.4.1. DNA Preparation for Exo III Digestion. This method was used to prepare a series of systematically deleted fragments. It is based on the enzymatic properties of Exonuclease III (Exo III), a 3' exonuclease specific for double-stranded DNA. Exo III can initiate digestion at blunt ends or ends with a 5' overhang, but cannot efficiently initiate digestion at a 3' overhanging end. For this purpose, primer MZ-Ex1 was created (Table 3). A *NotI* restriction site serving as the 5' overhang was followed by a short spacer to increase the efficiency of cutting. An *SphI* site created a 3' overhang and effected a stop for the Exo III. A *HindIII* site served as a cloning site into the pME1 vector. A second primer, MZ-Ex2 (Table 3), was created in the 5'→3' direction. The resulting, approximately 300 bp long PCR-fragment was digested with *NcoI* and *HindIII* and ligated into pME1 which had also been digested with *HindIII* and *NcoI* yielding pME1-1 (Fig. 2).

2.11.4.2. Exo III Digest. The resulting plasmid pME1-1 was double digested with *NotI* and *SphI*. Since Exo III is strongly inhibited by as little as 20 mM NaCl, the plasmid DNA had to be extracted with phenol and precipitated with ethanol following restriction digestion. The dried pellet was dissolved in 1 × Exo III ligation buffer [Tris-

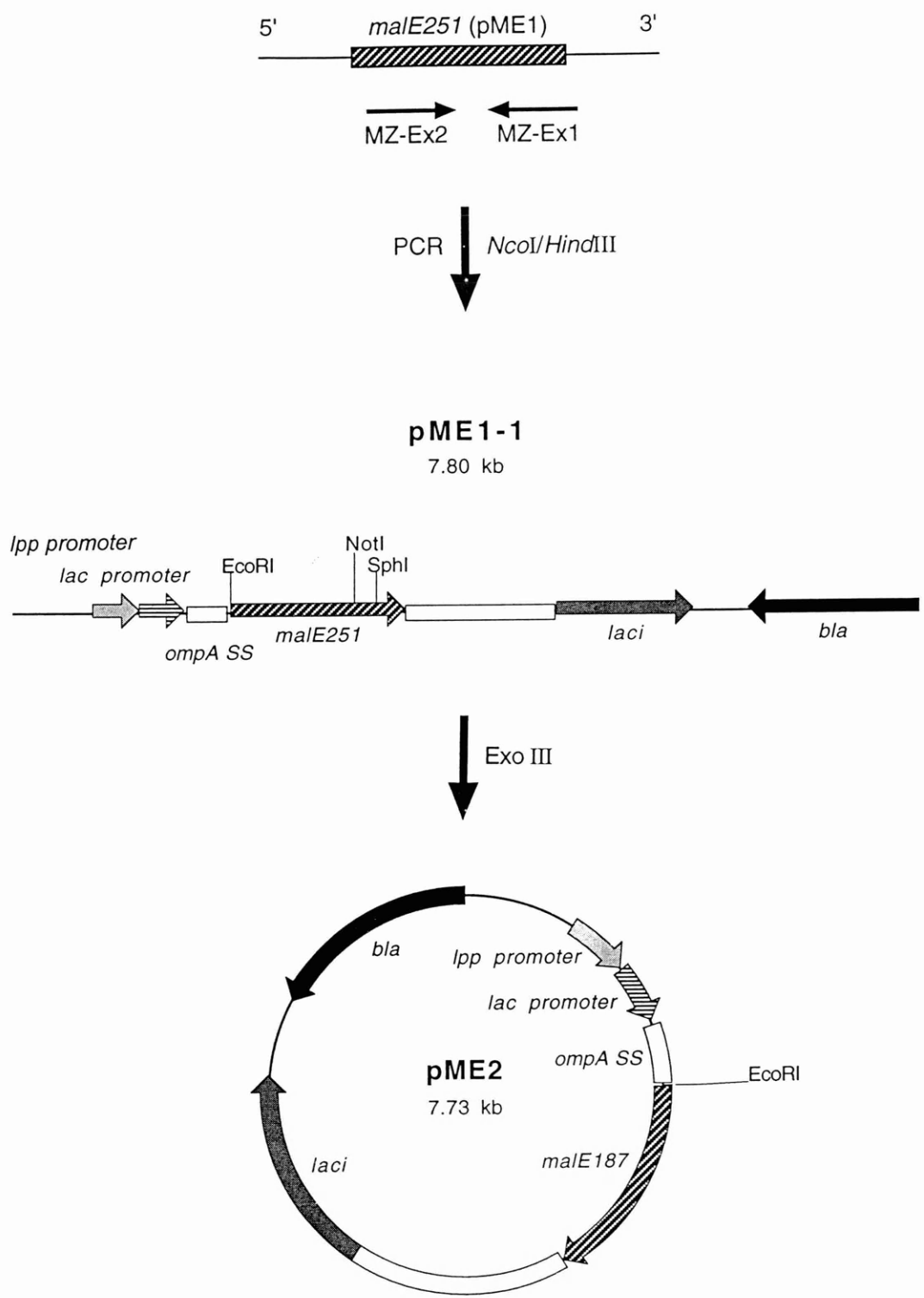


Fig. 2. Construction of plasmids pME1-1 and pME2. *bla*, β -lactamase (ampicillin-resistance gene); *lpp*, lipoprotein; *laci*, lac-repressor; *ompA*, outer membrane protein A; SS, signal sequence. For details, see text.

HCl (pH 7.5) 80 mM; dithiothreitol, 30 mM; MgCl₂, 20 mM] at a final concentration of 0.1 µg/µl. 100 µl of this solution were incubated with 1 µl Exo III (10 u) at 37°C for various times (5, 10, 15, 20, 30 min). The Exo III digest was inactivated at 70°C for 10 min and placed on dry ice.

2.11.4.3. S1 Nuclease Reaction. 10 µl of S1 nuclease buffer [sodium acetate (pH 4.6) 16 mM; NaCl, 400 mM; ZnSO₄, 1.6 mM; glycerol, 8%] plus 1 µl of S1 Nuclease (400 u) were added to each sample and incubated for 10 min at 37°C. The reaction was stopped by a pH-shift by adding 5 µl of S1 nuclease stop buffer [Tris-HCl (pH 8.0), 0.8 M; EDTA (pH 8.0), 20 mM; MgCl₂, 80 mM] to each sample. To verify the digestion, 4 µl of each sample were removed and run on a 0.4% agarose gel.

2.11.4.4. Creation of Blunt Ends and Re-Circularization. The DNA was made blunt ended with Klenow fragment (2 u) at 37°C for 60 min. The reaction was terminated by incubation at 70°C for 20 min. 1 µl (1 u) T4 DNA ligase was added to each sample which was incubated overnight at 15°C.

2.11.4.5. Characterization of the Exo III-Deleted pME1-1 Clones. 10 µl of DNA resulting from each of the various time points of the Exo III digest were transformed into strain MC4100rF' and plated on LB plates supplemented with glc and amp at 37°C. Five colonies were picked from each of five plates (resulting in 25 clones) and grown for 10 h. Cells were grown with supplementation of either glc/amp or IPTG/amp, and were subjected to immunoblot analysis with antibody directed against MalE. Further, a growth curve was prepared of the samples which showed a signal with the MalE antibody. The DNA of one selected clone (pME2) was sequenced.

2.11.5. DNA Sequencing

DNA sequencing was performed according to the method of Sanger *et al.* (1977) using double-stranded DNA. Sequencing was carried out with an Automated Laser Fluorescent (A.L.F.) DNA sequencer from Pharmacia Biotech. The system employs a non-radiochemical approach to sequencing; a primer labelled with fluorescein at its 5'-terminus is annealed to the template.

The concentration of the DNA was adjusted with distilled water to a concentration of 10 $\mu\text{g}/32 \mu\text{l}$ H₂O. After the addition of 8 μl NaOH (2 M), the mix was left for 10 min at room temperature. 7 μl of 3 M sodium acetate (pH 4.8) plus 4 μl of distilled water were added, followed by 120 μl 100% ethanol. The tubes were placed on dry ice for 30 min. The precipitated DNA was collected by centrifugation in an Eppendorf centrifuge at 14,000 rpm for 10 min. DNA was dried and resuspended in 11 μl distilled water. Primer annealing and sequencing reaction were performed with the "Auto Read Sequencing Kit" from Pharmacia following the instructions. The probes were run on an increased resolution gel (Table 6) using 0.35 mm spacers. The running conditions were according to the Pharmacia protocol.

Table 6: Sequencing Gel

Component	Amount
Urea	21 g
10 \times TBE	6 ml
30% Acrylamide : bis acrylamide (29:1)	11 ml
10% Ammonium peroxodisulfate	0.15 ml
<i>N-N-N'-N'</i> -tetramethylethylenediamine (TEMED)	0.04 ml
H ₂ O	ad 50 ml

2.12. Immuno-Electron Microscopy

Immuno-electron microscopy was performed as described (Freudl *et al.*, 1985). For better differentiation of inner and outer membranes, cells were plasmolyzed with 20% sucrose, fixed with 2% formaldehyde plus 0.05% glutaraldehyde, and embedded in Lowicryl HM20 at low temperature (Carlemalm *et al.*, 1982). Ultrathin resin sections were treated with rabbit anti-OmpA or rabbit anti-MalE serum and protein A-13 nm gold complexes.

2.13. Construction of Plasmids

2.13.1 Construction of pAME

To construct this *ompA-malE* hybrid, plasmid pRD87 (Freudl *et al.*, 1985) was used. pRD87 is a derivative of pUC8 bearing the amp resistance gene *bla*. Expression of *ompA* is under the control of the *lac* regulatory elements. pRD87 was digested with *Bgl*III and treated with the Klenow fragment of DNA polymerase I. The *malE* fragment was obtained from a *Bgl*III/*Nde*I digest of plasmid pPD1. The resulting 1 kb fragment was treated with Klenow enzyme and cloned into pRD87 opened with *Bgl*III, leading to the 525-residue *ompA-malE* hybrid gene (Fig. 3).

2.13.2. Construction of pME1

The gene coding for the MalE-part of the MalE-OmpA hybrid was cloned into the pINompA-2 vector (Ghrayeb *et al.*, 1984) where it was fused to DNA encoding the OmpA signal sequence. Plasmid pPDI was digested with *Bgl*III and *Nde*I. The resulting fragment (1 kb) was treated with Klenow enzyme and cloned into the pINompA-2 vector. This vector was cut with *Hind*III and then treated with Klenow. The resulting pME1 plasmid had an approximate size of 8 kb (Fig. 4).

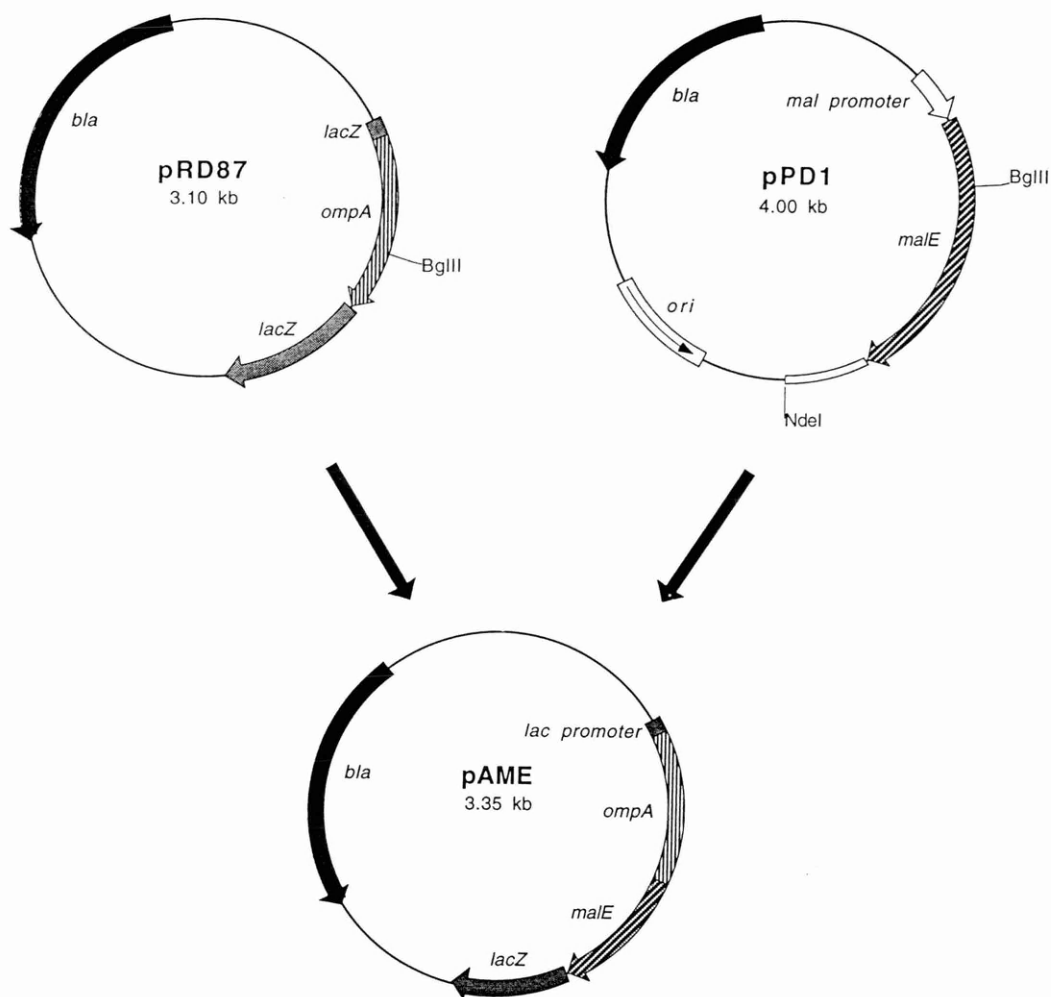


Fig. 3. Construction of plasmid pAME. *lacZ*, β -galactosidase.

2.13.3. Construction of pME1-cat

To select for IPTG-resistant *MalE*251 mutants, an operon fusion was constructed consisting of the truncated *malE* gene, followed by *cat*. Plasmid pME1 was digested with *Bam*HI and made blunt ended with Klenow enzyme. The *cat* resistance gene was obtained from a *Hind*III/*Xba*I digest of plasmid pDS5/3 (Stueber *et al.*, 1984) which yielded a 720 bp fragment carrying this gene. After treatment with Klenow enzyme, the 720 bp fragment was cloned into the *Bam*HI site of pME1 (Fig. 5).

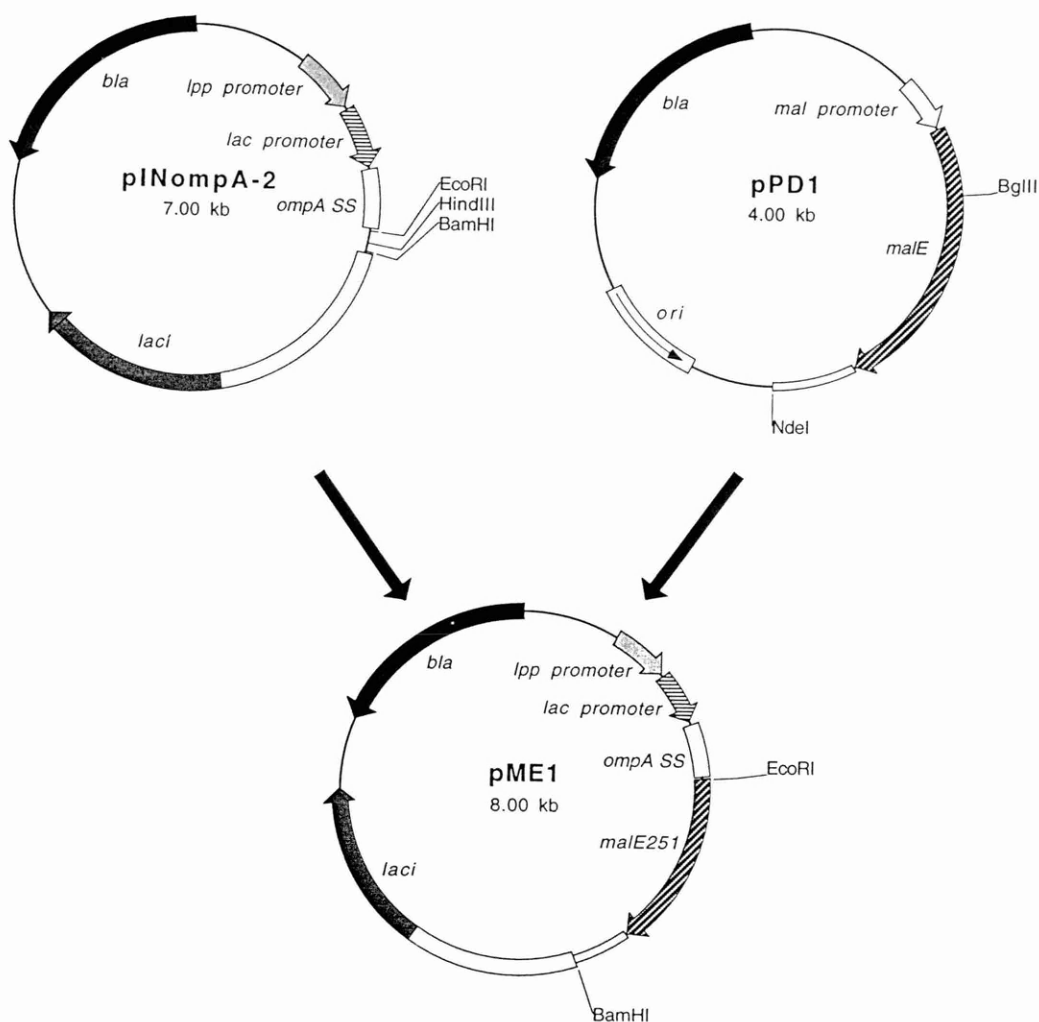


Fig. 4. Construction of plasmid pME1.

2.13.4 Construction of pK184G

A gene bank of chromosomal DNA from strain JM109 was kindly provided by Ralf Koebnik. In brief, the DNA was partially digested with *Sau3A*. The restriction fragments were separated by agarose gel electrophoresis, and an area of the gel containing fragments of a size range between 2 and 6 kb was eluted. These fragments were ligated into plasmid pK184 which had been linearized with *Bam*HI. (Fig. 6).

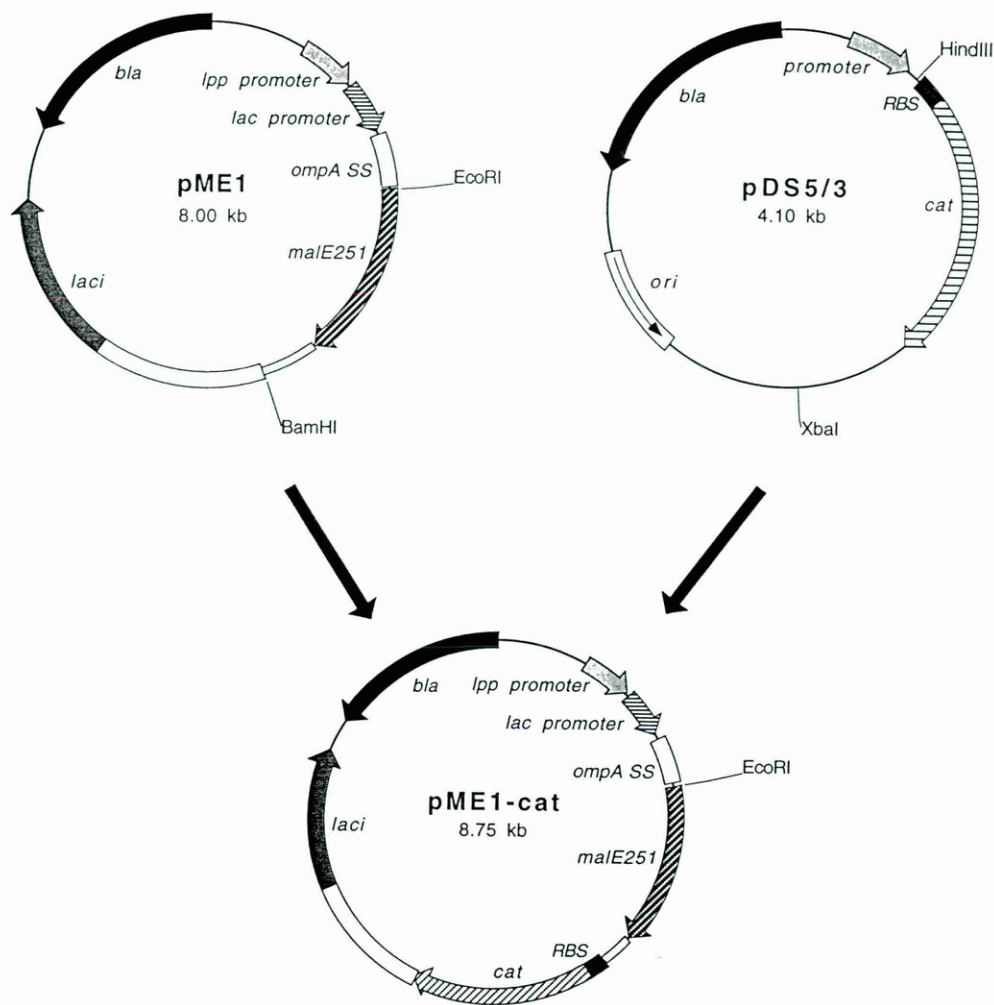


Fig. 5. Construction of plasmid pME1-cat. Operon fusion, consisting of the truncated *malE* gene (*malE251*) followed by the gene encoding chloramphenicol acetyltransferase (*cat*). RBS, ribosomal binding site. For details, see text.

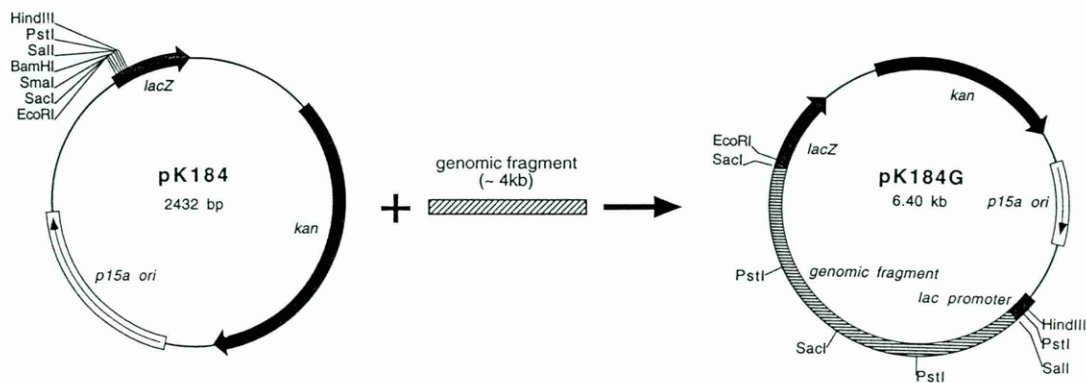


Fig. 6. Construction of plasmid pK184G. For details, see text.

2.13.5 Construction of pK184-Sac

Plasmid pK184-Sac is a subclone of pK184G. It was constructed to narrow down the region responsible for suppression of the toxicity caused by MalE251. For this purpose, pK184G was digested with *SacI*. The resulting 2.05 kb fragment was cloned into the *SacI* site of pK184 (Fig. 7).



Fig. 7. Construction of plasmid pK184-Sac.

2.13.6 Construction of pK184NE

To construct plasmid pK184NE, a *SalI* site was introduced at the 5' end of the partial *nlpE* gene via PCR. The resulting 140 bp fragment was digested with *SalI* and *HindIII*. It was then ligated into pK184 which had been digested with *SalI* and *HindIII*. The resulting plasmid pK184NE has a size of about 2.6 kb (Fig. 8).

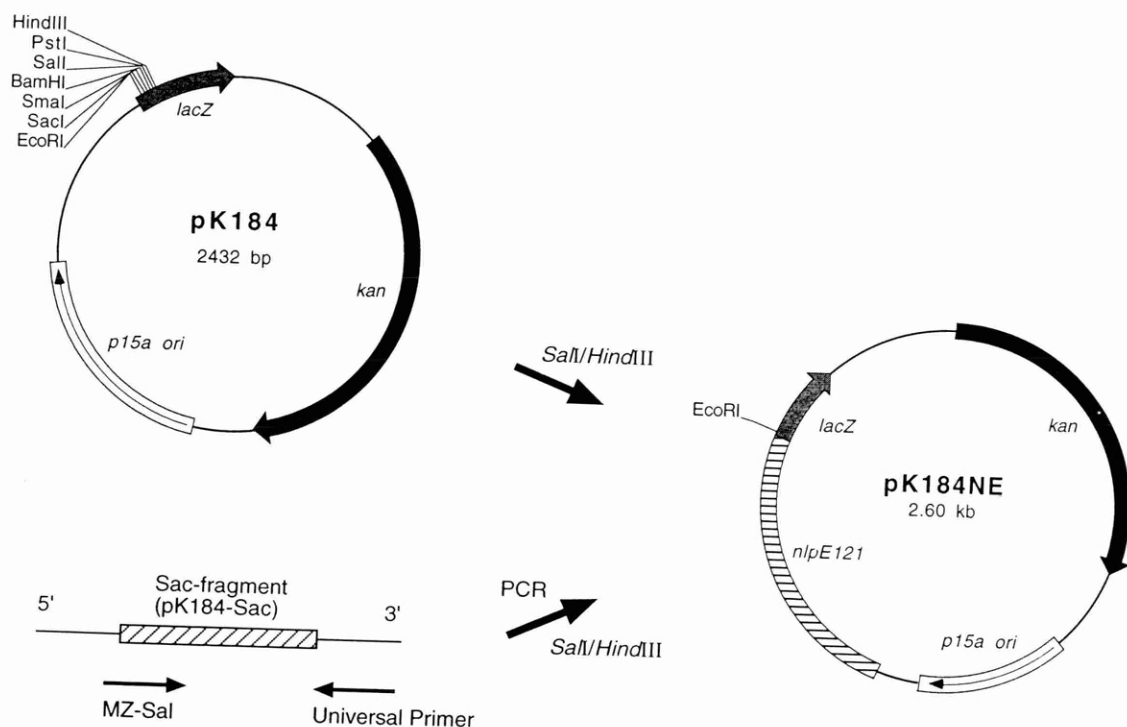


Fig. 8. Construction of plasmid pK184NE. This plasmid carries the gene *nlpE 121*, which codes for the N-terminal fragment of NlpE. MZ-Sal, primer used to introduce *SalI* site. For details, see text.

3. RESULTS

3.1 The OmpA-MalE Hybrid

Expression of the hybrid genes previously constructed by Klose *et al.* (1988) turned out to be toxic. For this reason, another hybrid gene coding for the first 274 residues of OmpA followed by the last 251 residues of MalE was constructed (see 2.13.2, "Materials and Methods: Construction of pME1"). Synthesis of this hybrid turned out to be even more toxic than the synthesis of the hybrids mentioned above. Cells lysed after just two to three cycles, if grown on glc plus IPTG at 37°C (Fig. 9).

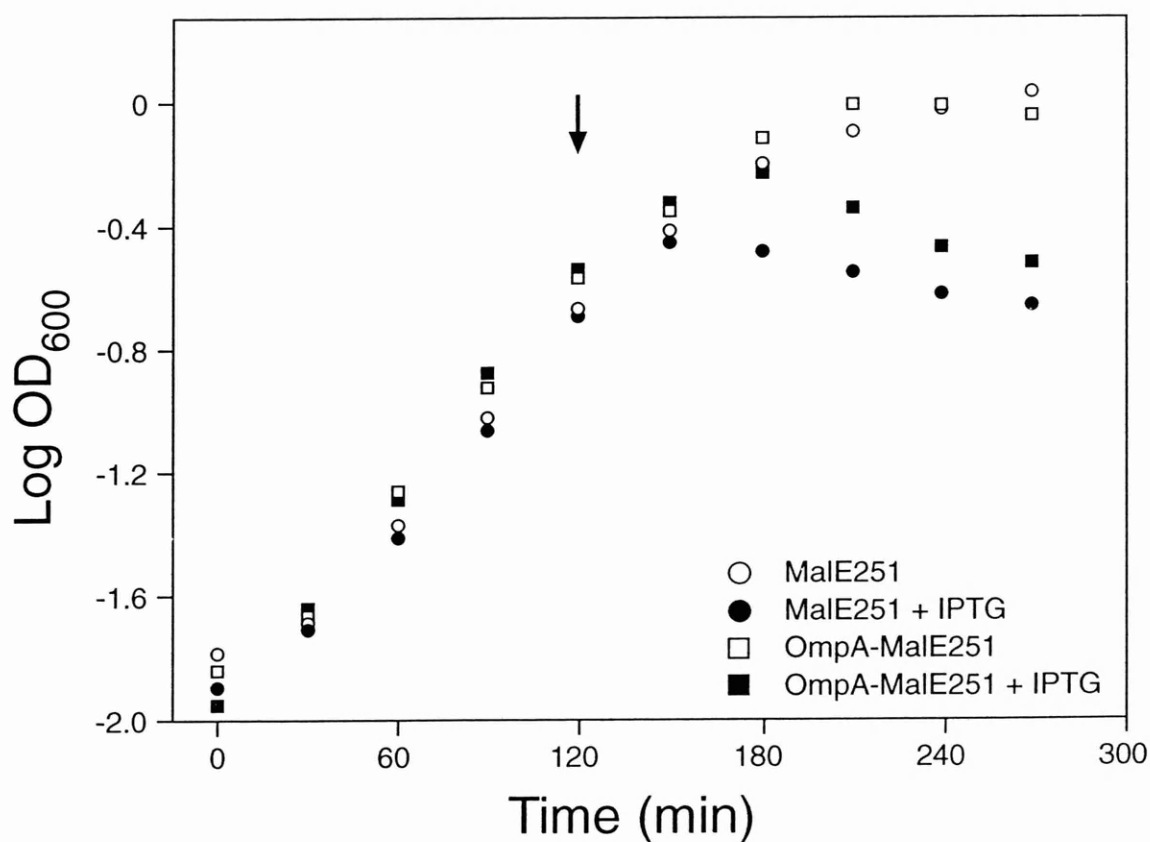


Fig. 9. Growth curves of cells carrying the plasmids coding for MalE251 or for the OmpA-MalE hybrid protein. Cells were pre-grown for 2 h with glc, before IPTG was added. The addition of IPTG is indicated by arrow.

3.2. The MalE Fragment

Synthesis of the OmpA part of the OmpA-MalE hybrid is not toxic. The cells can be grown on glc plus IPTG, and the OmpA fragment is correctly assembled into the outer membrane. Since nothing is known about the effect of synthesis of the MalE-part of the hybrid, the *malE*-fragment was cloned into a pIN-vector (Ghrayeb *et al.*, 1984) where it was attached to the gene coding for the OmpA signal sequence preceded by the *lac* promoter.

A polypeptide of the expected size was made (Fig. 10A, B), but expression of the truncated MalE fragment (MalE251) turned out to be even more toxic than that of the OmpA-MalE hybrid. Following the addition of IPTG, cells lysed after one to two generations at 37°C (Fig. 9).

3.3. Cellular Location of MalE251

One possibility for the observed toxicity of the MalE fragment is that this polypeptide causes jamming of the Sec translocation machinery. Thus, I examined whether the processed polypeptide was exported to the periplasm, or whether it remained at the plasma membrane. To answer this question, three sets of experiments were carried out: Western-blot analysis of spheroplasts; trypsin digestion of permeable cells; and immuno-electron microscopy.

To determine the subcellular location of the MalE fragment, cells containing plasmid pME1 were converted to spheroplasts (see 2.7, "Materials and Methods: Preparation of Spheroplasts") and incubated in the presence of IPTG for various times. To separate the cell envelope components from the periplasmic components, cells were centrifuged after incubation. The resulting pellet, as well as the TCA-precipitated

supernatant, were examined by Western blotting using MalE antibody. If the truncated MalE polypeptide remained with the plasma membrane, one would expect to detect most of the protein in the pellet. If, on the other hand, the MalE fragment is exported to the periplasm, the protein should be found mainly in the supernatant. As can be seen from Figure 11, almost all of the protein was detected in the supernatant. This shows that MalE251 did neither remain in the cytoplasm, nor did it get stuck at the cytoplasmic membrane, but was very efficiently translocated to the periplasm.

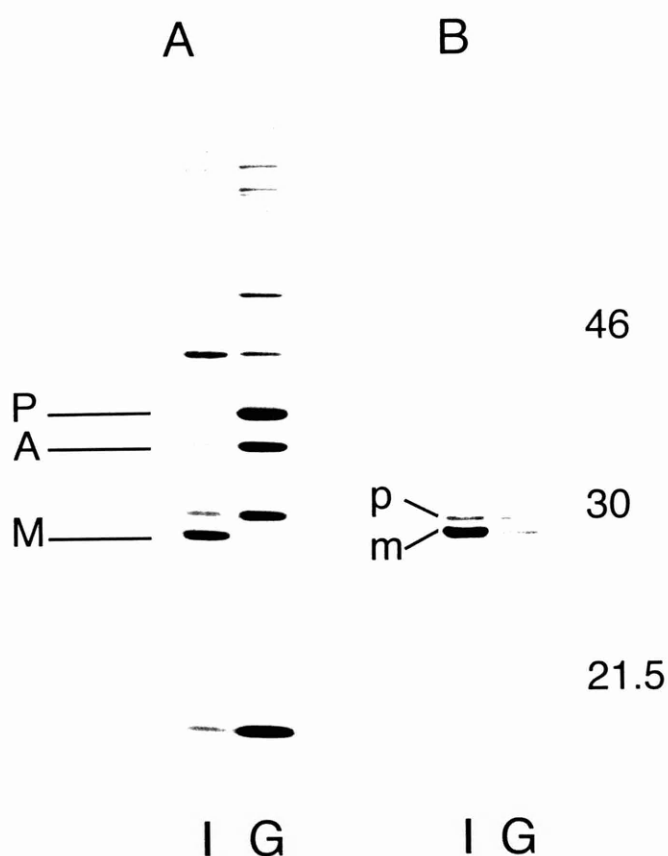


Fig. 10. A. Electrophoretogram of cell envelopes. A stained 12.5% SDS-polyacrylamide gel is shown. Strain MC4100 carrying plasmid pME1 was grown in the presence of glucose (G) or of IPTG (I) for 1 h. As can be seen in lane 1, the amount of OmpA and that of the porins is strongly reduced when the MalE fragment (MalE251) is induced. M, MalE251; P, porins; A, OmpA. B. Immunoblot analysis of whole cells, grown under the same conditions as in Fig. 10A, using rabbit anti-MalE antiserum. m, mature MalE251; p, precursor of MalE251.

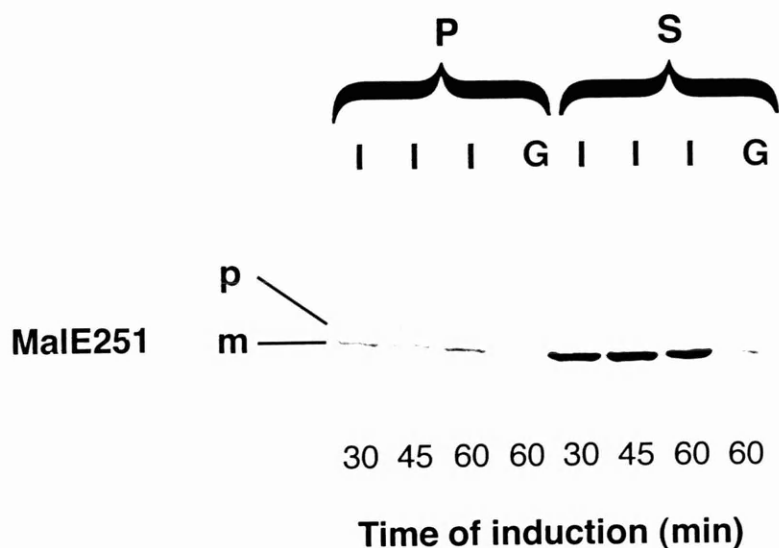


Fig. 11. Western-blot analysis of MalE251 synthesized in spheroplasts. Cells carrying plasmid pME1 were converted to spheroplasts and induced for various time periods. Regardless of the induction period, the MalE fragment is associated mainly with the TCA-precipitated supernatant (S) and not with the pellet (P). G, glucose; I, IPTG; m, mature MalE251; p, precursor of MalE251. For details see text.

The location of the MalE fragment in the periplasm was also confirmed by treatment of permeabilized cells with trypsin (see 2.10.6, "Materials and Methods: Trypsin Digestion"). The mature part of the MalE fragment was degraded by the protease so that no fragment could be detected electrophoretically (Fig. 12). This confirms that the truncated MalE polypeptide was located in the periplasmic space.

In addition, immuno-electron microscopy was performed to verify the results obtained with spheroplasts and trypsin-treated cells. The ultrastructural appearance of both the MalE-OmpA hybrid and the MalE fragment was identical (Fig. 13). They were located as aggregates in the periplasm, thus being in agreement with the above results.

3.4. Processing of the MalE Fragment

Only minute amounts of precursor of MalE251 accumulated. This has been demonstrated by Coomassie-stained SDS-polyacrylamide gel electrophoresis of

membrane preparations of MalE251, as well as by Western-blot analysis of whole cells and membrane preparations using an antibody directed against MalE (Fig. 10B). In the latter case, only very small amounts of the MalE251 precursor could be detected when large quantities of protein sample were used. To confirm that there was no significant accumulation of MalE251 precursor at the steady-state level, pulse chase experiments were performed (see 2.10.5, "Materials and Methods: Pulse-Chase Experiments and Immunoprecipitation"). For this purpose, exponentially growing cells were labelled with [³⁵S]methionine and subsequently chased with L-methionine. To test whether jamming of the export machinery occurred, samples were precipitated with either MalE- or with



Fig. 12. Trypsin sensitivity of the MalE fragment in permeabilized cells. Cells carrying plasmid pME1 were grown in the presence of IPTG (I), IPTG plus glucose (I/G), or glucose (G). While cells shown in the last three lanes were treated with trypsin (+), cells represented in the first three lanes remained untreated (-). Western-blot analysis using an antibody directed against MalE revealed that MalE251 is completely digested. M, protein molecular weight marker.

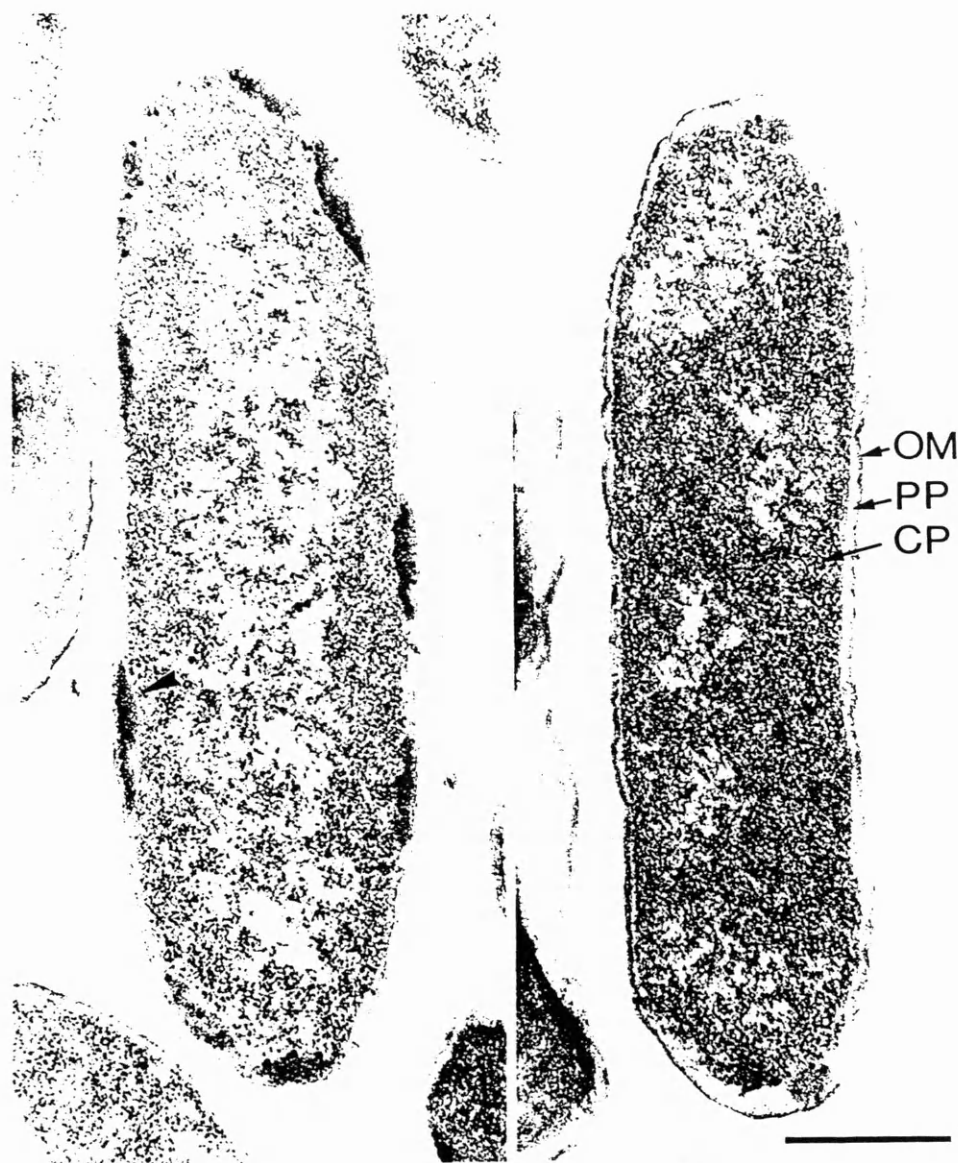


Fig. 13. Location of the OmpA-MalE hybrid protein (left) and the truncated MalE protein (right). Strain MC 4100 carrying the respective plasmid was grown for 1 h in the presence of IPTG. The ultrathin resin sections were treated with anti-OmpA antiserum (left) and anti MalE antibody (right). The antibodies were visualized with a protein A-13 nm gold complex. OM, outer membrane; PP, periplasm; CP, cytoplasm. Calibration bar, 0.5 μ m.

OmpA-antibody. These two immunoprecipitated polypeptides were analysed by SDS-polyacrylamide gel electrophoresis and autoradiography. As can be seen in Figures 14A and 14B, both polypeptides — chromosomally-encoded OmpA and plasmid-coded

MalE251 — were processed normally; there was no accumulation either of the precursor of the MalE fragment, or of another exported polypeptide, OmpA, when MalE251 was induced. Furthermore, the truncated MalE protein was stable and not degraded.

3.5. Selection for Nontoxic MalE251 Mutants

In an attempt to elucidate the cause(s) for the toxicity observed upon induction of the *malE* fragment, selection for mutants that are no longer lethal was carried out. To exclude, as far as possible, selection of mutants that no longer synthesize the protein, an operon fusion was constructed consisting of the truncated *malE* gene which is followed by the gene encoding chloramphenicol acetyltransferase (*cat*) (see 2.13.3, "Materials and Methods: Construction of pME1-cat"). Cells harboring this plasmid are

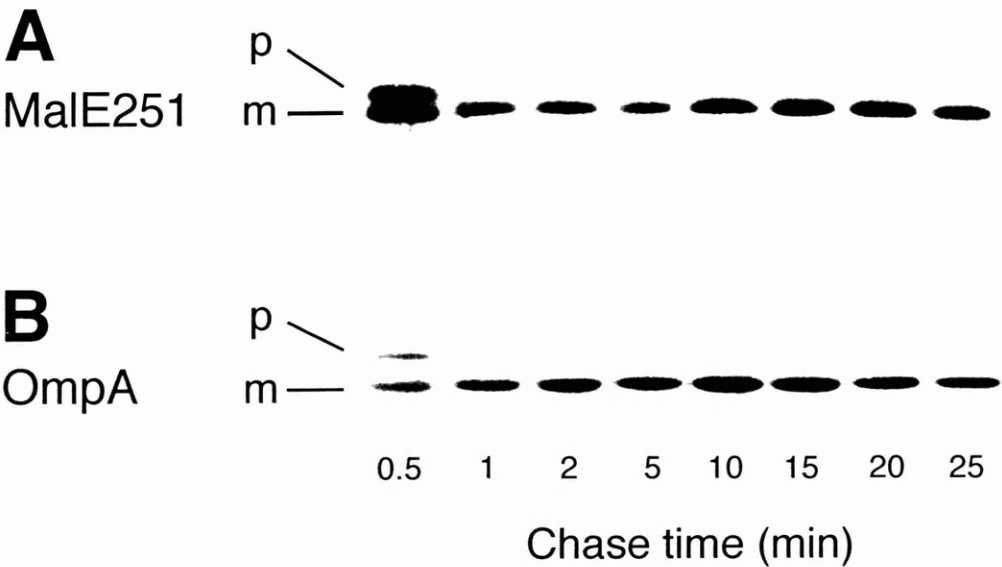


Fig. 14. Pulse-chase analysis of the processing of the MalE251 protein (A) and of the OmpA upon induction of MalE251 (B). Cells were immunoprecipitated with anti-MalE antibody (A) and anti-OmpA serum (B). An autoradiogram of an 12.5% SDS polyacrylamide gel is shown. p, precursor; m, mature protein.

not only resistant to amp, but also to chloramphenicol. Since both the truncated *malE* and the *cat* gene are controlled by the *lac* promoter, synthesis of MalE251 must also be accompanied by resistance to chloramphenicol.

Nine mutants of independent origin were analysed which were resistant to IPTG and still produced the MalE fragment (see 2.9, "Materials and Methods: Selection for IPTG-Resistant Mutants"). Three of these mutants were conditional, being able to grow in the presence of IPTG at 28°C, but not at 42°C. Plasmids were prepared from all nine mutants. These plasmids were transformed into strain MC4100rF', and cells were plated on LB plates which were supplemented with glc, IPTG, amp, and chloramphenicol. Only three of the mutants (two non-temperature-sensitive mutants and one temperature-sensitive mutant) were able to grow under these conditions. Thus, while two of the non-temperature-sensitive mutants and one temperature-sensitive mutant were plasmid-coded, all other mutants were chromosomal.

Examination of the plasmid-coded mutants appeared to be the most promising approach. The question was whether the absence of toxicity was due to a mutation in the *malE251* sequence, or to a gene-dosage effect. To answer this question, the *malE251* regions including the *ompA* signal sequence of all three plasmids (pME1-cat1, pME1-cat2, and pME1-catT3) were sequenced (see 2.11.5, "Materials and Methods: DNA Sequencing"). In no case, however, had a mutation in the coding region occurred. Absence of toxicity in the plasmid-coded mutants was, therefore, most likely due to reduced synthesis of the MalE251 protein. Western-blot analysis supported this notion, as can best be seen in the temperature-sensitive mutant T3. At the non-permissive temperature of 42°C, a significantly larger amount of the MalE251 protein was synthesized than was the case at the permissive temperature of 28°C (Fig. 15).



Fig. 15. Western-blot analysis of the temperature sensitive mutant, T3. At the non-permissive temperature of 42°C, a significantly higher amount of MalE251 is produced than was the case at the permissive temperature of 28°C. In contrast to the lower temperature, at 42°C no chromosomally encoded MalE can be detected with an antibody directed against MalE. G, glucose; I, IPTG; m, mature MalE251; p, precursor of MalE251.

3.6. Modification of MalE251

3.6.1. C-terminal Deletion of MalE251 Results in Loss of Toxicity

Since the lack of toxicity observed upon induction of the mutant MalE251 plasmids (pME1-cat1, pME1-cat2, pME1-catT3) appeared to be due to the fact that these mutants simply produced less of the toxic MalE251, the following question arose: can the *malE* gene be altered such that its product is no longer lethal to the cell? To answer this question, I deleted stepwise parts of the gene encoding the MalE251 C-

terminus. For this purpose, I made use of the properties of Exonuclease III (Exo III).

An appropriate set of primers (MZ-Ex1 and MZ-Ex2) was created to produce a 5'-overhang, where Exo III can initiate digestion, and an Exo III-resistant 3'-overhang (for details, see 2.11.4, "Materials and Methods: Exonuclease III Deletions"). The amplified DNA was cloned into pME1 resulting in pME1-1 (Fig. 2). After a *NotI/SphI* digest, pME1-1 was treated with Exonuclease III for various times (5, 10, 15, 20 and 30 min). Subsequent to inactivation of Exo III, S1 Nuclease was added to remove the remaining single-stranded DNA tails. The degree of the DNA digest was analysed on an agarose gel.

After inactivation of S1 Nuclease, Klenow DNA Polymerase was added to polish the ends, which then were ligated to circularize the deletion-containing vectors. The five ligation mixtures were transformed into strain MC4100rF'. Each of the successive time points yielded a collection of subclones containing clustered deletions. Five colonies were picked from each plate obtained at a given time point. The resulting 25 clones were grown in the presence of IPTG. Western-blot analysis identified five clones to be immunopositive for MalE. One of these five clones showed no signs of toxicity when synthesis of the MalE polypeptide fragment was induced (Fig. 16). This clone, pME2, was further analysed.

The new clone pME2 lacked the last 66 base pairs. This result was in agreement with the size of the protein band of the mature protein, which exhibited in Western blots an approximate size of 25 kDa (Fig. 17). Furthermore, Western-blot analysis showed that, as in the case with MalE251, also with the new protein (MalEME2) no accumula-

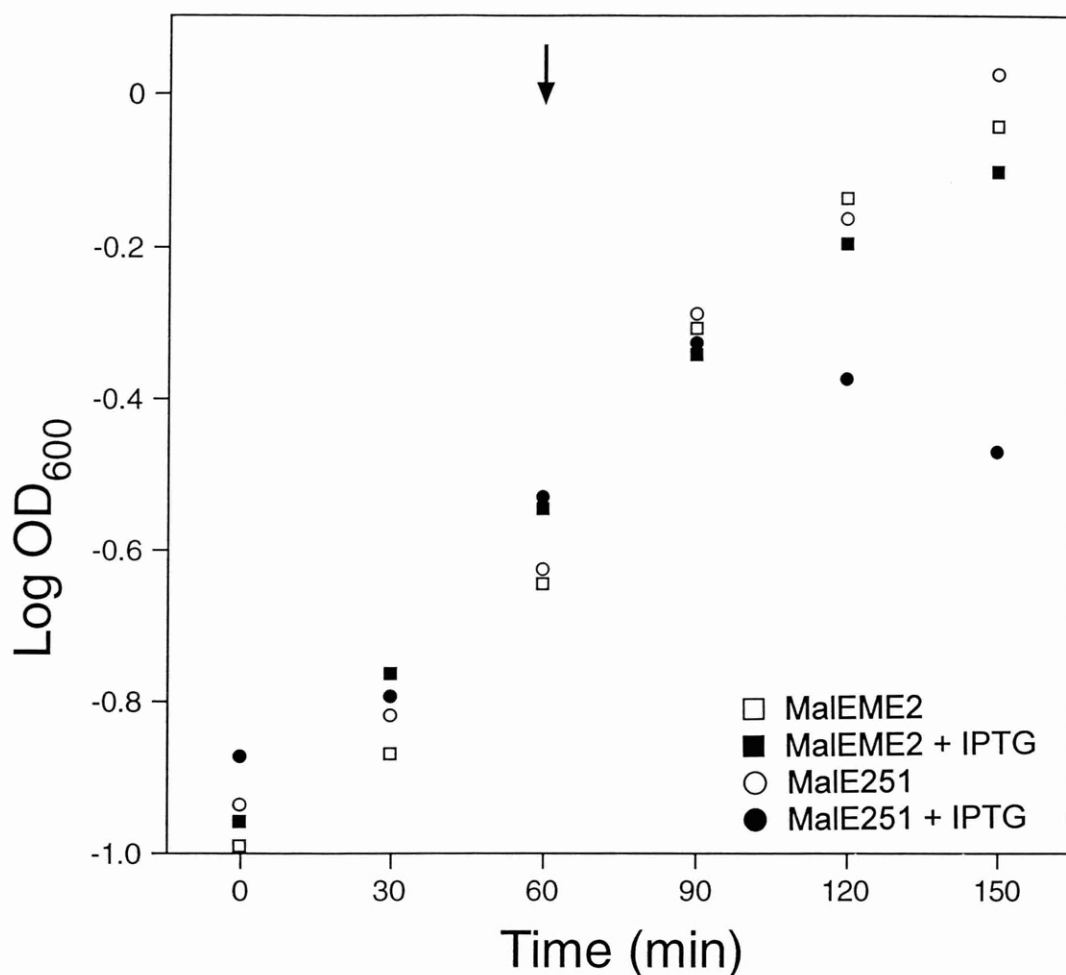


Fig. 16. Growth curves of cells carrying plasmids coding for MalE251 and MalEME2, respectively. Cells were pre-grown for 1 h before IPTG was added to the medium. The addition of IPTG is indicated by arrow.

tion of the precursor occurred. The amount of the mature protein (MalEME2) produced upon induction was only slightly smaller than that of MalE251 (Fig. 17).

3.6.2. MalEME2 is Insensitive to Trypsin Digestion

To learn more about the behaviour of the C-terminally deleted MalE fragment, a trypsin digest with permeabilized cells was performed. MalE251 served as a control.

While the mature part of MalE251 was completely digested by the protease at concentrations of 0.1 mg/ml, so that no fragment could be detected electrophoretically, treatment with trypsin of MalEME2 reduced the amount of the mature protein only marginally (Fig. 18). This was true even when the trypsin concentration used was fivefold of that used with MalE251. This result indicates that MalEME2 was not freely accessible for trypsin. Thus, MalEME2 was either not (or only in minor amounts) secreted across the cytoplasmic membrane, or it formed inclusion bodies in the periplasm. The formation of periplasmic inclusion bodies by incorrectly folded MalE mutants has been described previously (Betton and Hofnung, 1996; Betton *et al.*, 1996).



Fig. 17. Immuno-blot analysis of whole cells using rabbit anti-MalE antiserum. G, glucose; I, IPTG; m, mature protein; p, precursor.

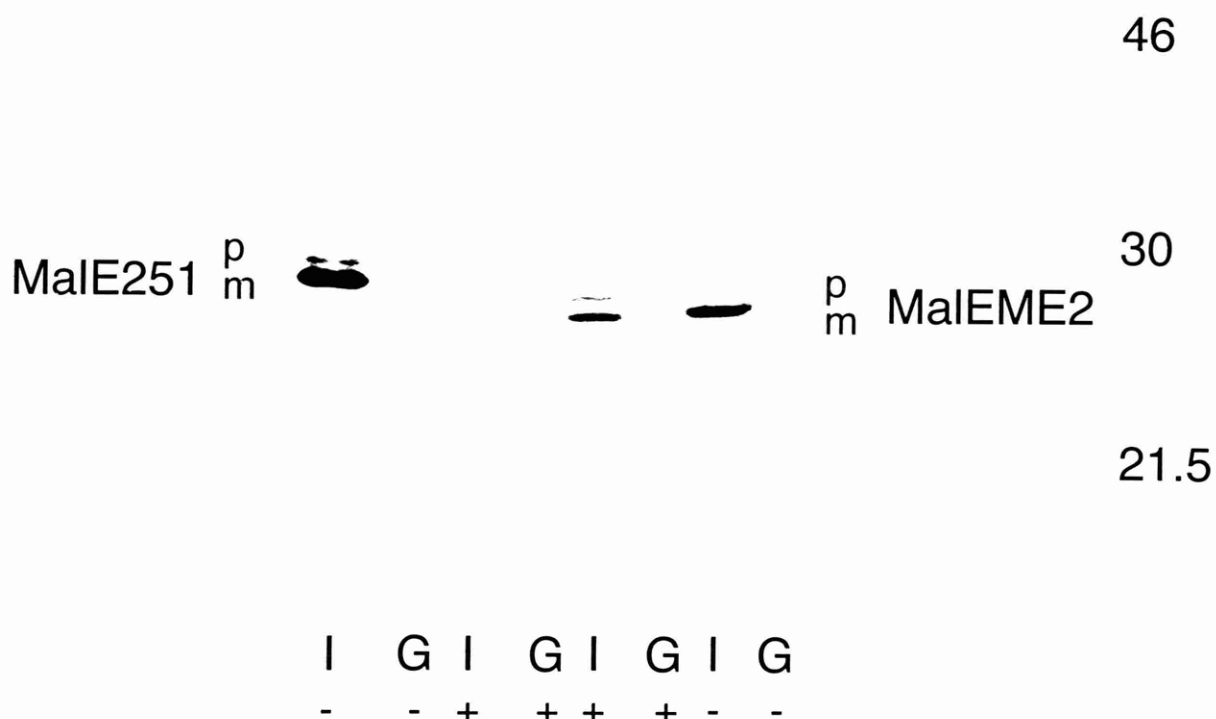


Fig. 18. Trypsin sensitivity of MalE251 and MalEME2 in permeabilized cells. Cells in lanes 3-6 were treated with trypsin (+), cells in lanes 1, 2, 7, and 8 remained untreated (-). Western-blot analysis using an antibody directed against MalE revealed that, while MalE251 is completely digested, treatment with trypsin reduced the amount of MalEME2 only marginally. I, IPTG; G, glucose; m, mature protein; p, precursor.

3.6.3. MalEME2 is Transported across the Plasma Membrane

To examine the location of MalEME2 further, cell fractionation was performed. These experiments, followed by Western blot using an antibody directed against MalE, revealed that MalEME2 was neither located in the cytoplasm, nor was it freely available in the periplasm; rather, it was entirely recovered in the subcellular membrane fraction (Fig. 19).

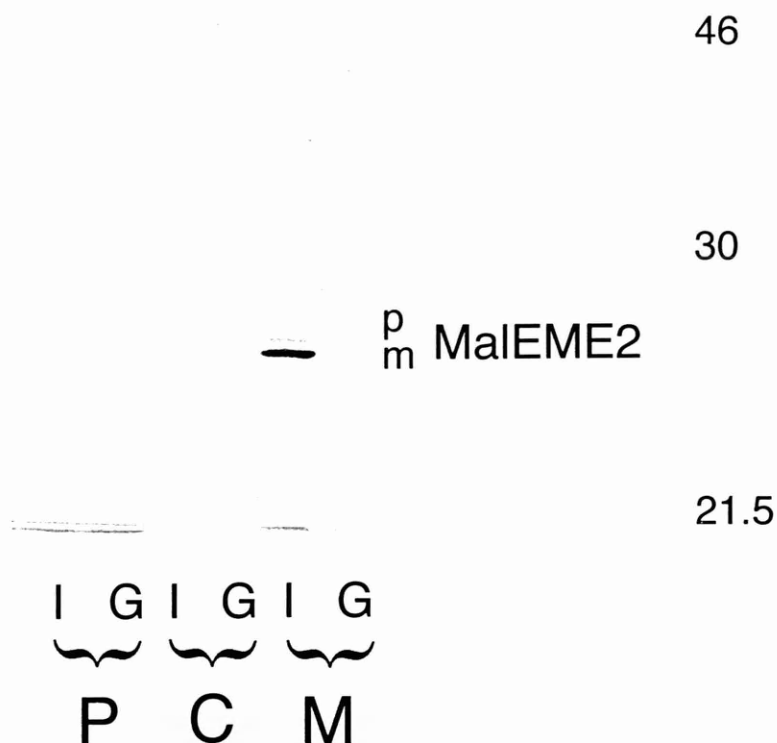


Fig. 19. Cell fractionation followed by Western blot using an antibody directed against MalE revealed that MalE251 is entirely recovered in the subcellular membrane fraction, M. P, periplasmic fraction; C, cytoplasmic fraction; p, precursor of MalE251; m, mature MalE251.

3.7. Multicopy Suppression

3.7.1. Selection and Identification of the Suppressor

One approach which has been successfully used with other systems (Snyder *et al.*, 1995) is to search for suppressors of toxicity. For this purpose, chromosomal DNA was partially digested with *Sau3A*. The digest was fractionated on an agarose gel, and fragments with a size of 2-6 kb were cut out and eluted. These fragments were cloned into the *Bam*HI site of pK184 (Jobling and Holmes, 1990). This plasmid is a low-copy-number vector with a p15a replicon; it carries the resistance gene *kan* which specifies the enzyme aminoglycoside phosphotransferase. This vector was chosen, since it is not possible to clone some genes (e.g. *ompA*) into high-copy-number vectors.

The library was transformed into strain MC4100rF' which carried plasmid pME1-cat. Each transformation was plated on two different media: Firstly; LB, amp, kan, glc; this resulted in approximately 9,000 transformants. Secondly; LB, amp, kan, IPTG; this resulted in one clone. The plasmid of this clone (pK184G), carrying a 4 kb genomic insert, was transformed into strain MC4100rF' which had been transformed with pME1. Again, the two different media (see above) were used. In the first case, with glc, 75 colonies were obtained. In the second case, with IPTG, 15 colonies appeared. This transformation was repeated, leading to the same ratio. Twenty clones obtained on the medium containing glc were plated on medium containing IPTG, and all of them grew on this medium. The difference in the number of clones with the two different media is, therefore, due to a decreased efficiency of plating when IPTG was present.

To narrow down the area of the 4 kb genomic insert which caused suppression, a restriction analysis was performed. *SacI* and *PstI* were found to have unique sites in the insert. They were used to dissect the 4 kb fragment further. A digest of pK184G with *SacI* resulted in a 1.8 kb fragment (*Sac*-fragment) while a digest with *PstI* produced a 2.2 kb fragment (*Pst*-fragment). The two fragments were subcloned into plasmid pK184 which was digested with *SacI* or *PstI*, respectively. The resulting plasmids (pK184-*Sac* and pK184-*Pst*) were independently transformed into strain MC4100rF' into which plasmid pME1-cat had been introduced. Induction with IPTG showed that the biological activity is located on the *Sac*-fragment.

The 1.8 kb insert was sequenced. This sequence revealed the existence of two complete open reading frames (ORFs) (Fig. 20). One of them, ORF140, codes for a protein involved in copper tolerance in *E. coli* (Gupta *et al.*, 1995). The other complete

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1      GTTACAATGGCCTCCTGATTCGAAAGGAGTTTTCTTATGGCGCTTAAAGCGACAATTTATAAAGCGACGGTTAATGTGGCC
      YaeQ-M  A L K A T I Y K A T V N V A

82     GATCTCGACCGCAACCAGTTTCTCGATGCCTCTCTGACGCTGGCGGCCATCCTTCAGAAACCCAGGAGCGTATGATGCTG
      D L D R N Q F L D A S L T L A R H P S E T Q E R M M L

163    CGTTTGCTGGCGTGGCTGAAATATGCTGATGAACGTCGTCAATTTACCCGTGGTTTGTGTGCCGATGATGAGCCGGAAGCG
      R L L A W L K Y A D E R L Q F T R G L C A D D E P E A

244    TGGCTGCGTAACGATCACCTGGGCATTGATTTGTGGATTGAGCTGGGGCTGCCGGATGAGCGGCGGATTAAGAAAGCCTGC
      W L R N D H L G I D L W I E L G L P D E R R I K K A C

325    ACCCAGGCCGCGAGAAGTGGCGCTGTTTACCTATAATAGTCGGGCGGCGCAAATCTGGTGGCAGCAAAATCAGAGCAAATGT
      T Q A A E V A L F T Y N S R A A Q I W W Q Q N Q S K C

406    GTGCAGTTTGCCAACTCTTCCGCTCTGGTATCTGGACGATGAACAACTGGCGAAAGTAAGCGCCTTTGCCGATCGTACCATG
      V Q F A N L S V W Y L D D E Q L A K V S A F A D R T M

487    ACGCTGCAGGCAACGATTGAGGATGGCGTGATCTGGTTATCGGATGATAAGAATAATCTGGAAGTGAACCTAACCGCCTGG
      T L Q A T I Q D G V I W L S D D K N N L E V N L T G W

568    CAACAACCTTCATGA
      Q Q P S *

564    CTGGCAACAACCTTCATGATTGTGATTTCCTCGACATGTTGCTATTCCCGATGGTGAGCTTGAGATCACCGCCATTCGTGCG
      ORF140-M I V I S R H V A I P D G E L E I T A I R A

645    CAGGGCGCGGGCGGGCAGCATGTTAATAAGACCTCAACGGCTATTCATCTGCGTTTTGACATTCGGGCGTCCAGCCTGCCA
      Q G A G G Q H V N K T S T A I H L R F D I R A S S L P

726    GAGTATTACAAAGAGCGTCTGCTCGCCGCGAGCCATCATTTGATCAGCAGTGATGGCGTGATTGTCATTAAGGCACAGGAA
      E Y Y K E R L L A A S H H L I S S D G V I V I K A Q E

807    TACCGCAGTCAGGAAGTGAACCGCGAAGCAGCTCTGGCCCGGCTGGTGGCTATGATTAAAGAATTAACAACAGAAAAAAA
      Y R S Q E L N R E A A L A R L V A M I K E L T T E K K

888    GCCCCAGACCCACGCGGCCACCCGTCATCGAAAGAGCGCAGGCTGGCATCGAAAGCACAAAAATCAAGCGTGAAGGCG
      A R R P T R P T R A S K E R R L A S K A Q K S S V K A

969    ATGCGCGGCAAAGTGCGCAGCGGTCGCGGAATAAA
      M R G K V R S G R E *

1003   AAGAAGGAATGGATGGTGAAAAAGCGATAGTGACGCGATGGCTGTAATCAGCCTCTTTACTCTGATGGGATGTAATAAT
      NlpE121-M V K K A I V T A M A V I S L F T L M G C N N

1084   CGGGCCGAAGTCGATACGCTTTCTCCGGCGCAGGCTGCCGAAGTGAACCGATGCCGCAAAGTTGGCGCGGCGTGCTGCCG
      R A E V D T L S P A Q A E L K P M P Q S W R G V L P

1165   TGTGCCGATTGCGAAGGAATCGAAACCTCTCTGTTCTCGAAAAAGACGGAACATGGGTGATGAATGAGCGTTATCTCGGT
      C A D C E G I E T S L F L E K D G T W V M N E R Y L G

1246   GCTCGTGAAGAACCTTCCTCCTTCGCTTCTACGGTACATGGGCGCGAACCCTGACAAGCTGGTATTAACCGACAGCAAA
      A R E E P S S F A S Y G T W A R T A D K L V L T D S K

1327   GGTGAAAAGTCATATTATCGGGCGAAAGCGATGCGCTGGAGATGCTCGATCCCGGGTACCGAGCTCCAGCTTTTGTTC
      G E K S Y Y R A K G D A L E M L D P R V P S S S F C S

1408   CTTTAG
      L *

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Fig. 20. Sequence analysis of the fragment causing suppression of the toxicity caused by MalE251 revealed the existence of two complete open reading frames (YaeQ and ORF140) and a partial open reading frame containing the 121 N-terminal residues of the 236 residue NlpE (NlpE121). The putative Shine-Dalgarno ribosomal-binding sites are underlined. The vertical arrow indicates the end of identity between NlpE and NlpE121. *, STOP codon.

ORF is called YaeQ (Blattner *et al.*, 1997); its function is not known. Downstream from ORF140, a partial ORF was located (Fig. 20).

The partial ORF contained 121 (out of a total of 236) *N*-terminal residues of New lipoprotein E (NlpE). The precursor of the 25,829 Da-NlpE possesses an amino-terminal signal sequence. It was recently found that overproduction of the complete polypeptide is able to suppress the toxicity of the periplasmic LamB-LacZ-PhoA fusion protein, as well as the extracytoplasmic toxicity conferred by a mutant outer membrane protein, LamBA23D (Snyder *et al.*, 1995). High-level synthesis of the LamB-LacZ-PhoA fusion protein during growth in the presence of its inducer maltose exerts a pronounced toxicity. The tripartite protein forms high-molecular-weight aggregates in the periplasm. Although the cause of the toxicity is still unknown, it is thought that LacZ titrates some essential periplasmic factors (Snyder and Silhavy, 1995). LamBA23D contains a defect in the signal sequence cleavage site which causes poor processing of the LamBA23D precursor. Accumulation of unprocessed LamBA23D in the bacterial envelope leads to a permeability defect (Carlson and Silhavy, 1993). In both of the above mutants, the LamB-LacZ-PhoA fusion protein and the LamBA23D protein, suppression occurred by activation of the Cpx two-component signal transduction pathway which controls the expression of the periplasmic protease DegP, as well as of other factors that can combat certain types of extracytoplasmic stress. The Cpx two-component signal transduction pathway will be discussed in more detail below.

To examine whether the partial *nlpE* is responsible for the observed suppression of the toxicity caused by induction of synthesis of the MalE fragment, a subclone of pK184-Sac was constructed (for details, see 2.13.5, "Materials and Methods: Construction of pK184-Sac"). A *SalI* site was introduced at the 5'-end of the partial

nlpE gene. The *Hind*III site of the vector following the *nlpE* fragment was used as the 3'-restriction site. The resulting fragment was ligated into pK184 which was digested with *Sal*I and *Hind*III, resulting in plasmid pK184NE (see Fig. 8). pK184NE was transformed into strain MC4100rF' which carried pME1-cat. Indeed, the *nlpE* fragment proved to be responsible for mediating suppression (Fig. 21). Sequence analysis of pK184NE confirmed that the construct possessed the gene fragment coding for the first 121 amino acid residues of the NlpE polypeptide.

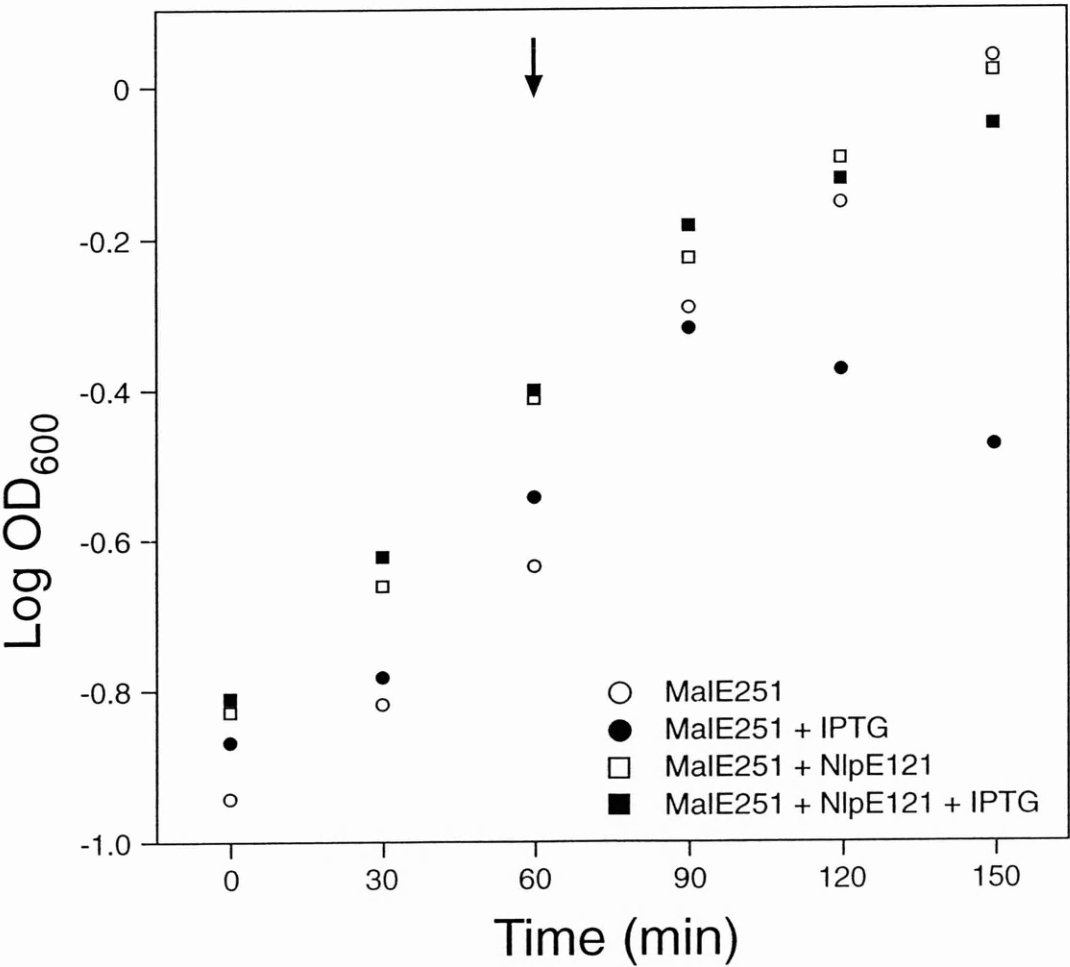


Fig. 21. Growth curves of cells carrying either plasmid pME1 (coding for MalE251) only, or containing the additional plasmid pK184NE (coding for NlpE121). Cells were pre-grown for 1 h, before IPTG was added. The addition of IPTG is indicated by arrow.

3.7.2. Proposed Mechanism of Suppression

How may the NlpE fragment act? It has been reported previously that overproduction of NlpE exerts a strong signal to induce expression of the periplasmic protease DegP (Danese *et al.*, 1995). This protein is known to be involved in the degradation of abnormal periplasmic proteins (Strauch and Beckwith, 1988). Western-blot analysis showed that, in contrast to the MalE251 precursor protein, the amount of the mature MalE251 polypeptide was drastically reduced in the presence of the NlpE fragment (Fig. 22). This strongly indicated that the mature part, which was located in the periplasm (see above), was digested by DegP and thus abolished the toxicity.

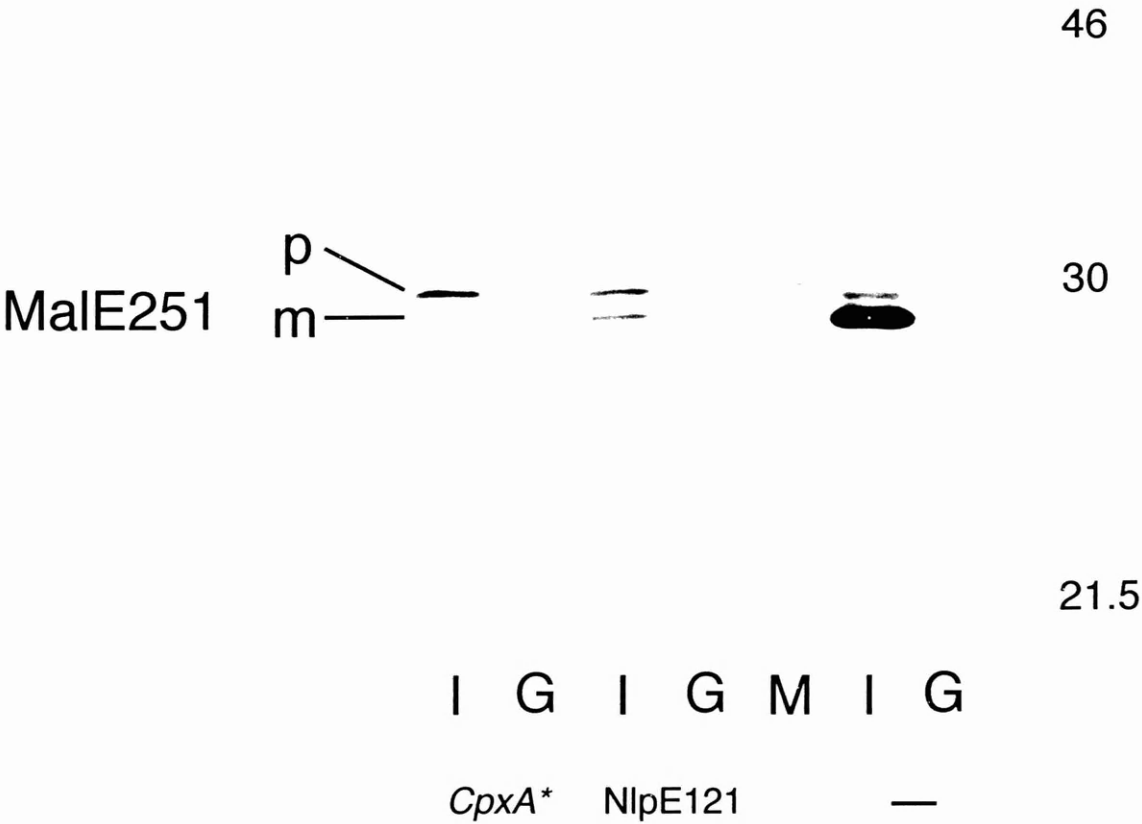


Fig. 22. Western-blot analysis of MalE251 grown either in a *cpxA** background in cells carrying the additional plasmid pK184NE, or in cells carrying pME1 only. In the first two cases, almost no mature MalE251 (m) can be detected, while the amount of the precursor (p) remains unaffected. I, IPTG; G, glucose. M, protein molecular weight marker.

3.7.3. Mutations in *cpxA* Stimulate Transcription of *degP*

Danese and co-workers (1995) discovered a class of suppressors that are able to combat the toxic effects exerted by the LamB-LacZ-PhoA fusion protein and LamBA23D. These suppressor mutations encode activated alleles of the previously characterized *cpxA* gene (McEwen and Silverman, 1980) and were named *cpxA**. The *cpxA* gene encoding the membrane sensor component of the Cpx two-component signal transduction system was identified more than 15 years ago (McEwen and Silverman, 1980a, b; Silverman 1982; Albin and Silverman, 1984) and sequenced in 1988 (Weber and Silverman, 1988). The CpxA polypeptide is an inner membrane protein consisting of a cytoplasmic and a periplasmic domain joined by two transmembrane segments. The hypothesis that CpxA functions as a sensory polypeptide is supported by structural similarities between the CpxA protein and the chemosensory transducers (Krikos *et al.*, 1982; Weber and Silverman, 1988). These similarities extend to overall size, cellular location, number of membrane-spanning segments, and also to the sizes of their extracellular and intracellular domains. A further similarity is the extremely low number of cysteine residues. These results suggest that CpxA, like chemosensory transducers, senses changes in the medium surrounding the cells and communicates this information to its cognate response regulator CpxR. The *cpxR* gene codes for the protein acting as the cognate regulator of the membrane sensor, CpxA. *cpxR* is located immediately upstream of the *cpxA* locus (Dong *et al.*, 1993).

Since various *cpxA** suppressor mutations lead to a drastic decrease of toxicity caused by the LamB fusion proteins (Danese *et al.*, 1995), the question arose whether a similar effect could be observed with the MalE251 polypeptide. Therefore, I transformed plasmid pME1 into a *cpxA** strain (*cpxA17*). After induction with IPTG,

no toxicity could be observed. Furthermore, the optical density (OD₆₀₀) of the induced cells was comparable to that of the non-induced ones. The lack of toxicity was also confirmed by using solid medium containing IPTG which does not allow colony formation of cells containing the MalE fragment only. These results are consistent with the observations of Danese *et al.* (1995). In the latter study, *cpxA** alleles completely abolished the extracytoplasmic toxicity conferred by the LamB model system. Moreover, it could be demonstrated that these suppressors function by increasing the expression of the periplasmic protease DegP (Cosma *et al.*, 1995), which enhances the degradation of the LamB-LacZ-PhoA protein. This appears also to be true for the MalE251 polypeptide. As in the presence of NlpE121, Western-blot analysis demonstrated the mature MalE251 protein to be almost completely absent when MalE251 was expressed in a *cpxA** background (Fig. 22).

3.7.4. Induction of DegP Does Not Always Lead to Suppression of Toxicity

Overproduction of outer membrane proteins or high temperatures or high concentrations of ethanol are stress factors (Neidhardt and VanBogelen, 1987) known to increase the rate of synthesis of DegP (Mecsas *et al.*, 1993; Danese *et al.*, 1995). However, it has been shown that overproduction of the outer membrane protein OmpF does not suppress the toxicity caused by the tripartite LamB-LacZ-PhoA strains (Snyder *et al.*, 1995). This holds true, although synthesis of DegP is very strongly induced. Thus, I tested high concentrations of ethanol (5% and 10%) as well as high temperature (up to 45°C). Under each of the two environmental stress conditions, I could not observe a reduction of toxicity, when MalE251 was expressed. In contrast, cells in which MalE251 was induced with IPTG could not cope at all with an ethanol

concentration of 10%, and grew very poorly at concentrations of 5%. These results indicate either that induction of DegP by these mechanisms is not sufficient to combat the toxicity caused by expression of the MalE251 protein, or that another (possibly an additional) factor is necessary to abolish toxicity.

3.8. Influence of MalT Expression on the Toxicity Exerted by MalE251

The maltose regulon of *E. coli* contains seven operons which encode the proteins involved in the utilization of starch and its degradation products, such as maltose and maltotriose, as carbon and energy source (for review, see Schwartz, 1987). Expression of the maltose regulon is controlled by MalT. This protein is a transcriptional activator encoded by the *malT* gene (Cole and Raibaud, 1986). In the presence of maltotriose and ATP, MalT binds to a short asymmetrical sequence which is present in several copies in all MalT-dependent promoters; it increases the rate of open complex formation at these promoters. The activation of two of the promoters, *malEp* and *malKp*, also requires the cAMP receptor protein, CRP (Vidal-Ingigliardi and Raibaud, 1991).

Is there an influence of MalT expression on the toxicity exerted by MalE251? An activation of *malEp* could increase the amount of chromosomal MalE, which might down-regulate the amount of MalE251, thus reducing the toxicity caused by the latter protein. To answer this question, plasmid pOM25 (Cole and Raibaud, 1986) was transformed into strain MC4100rF', carrying pME1. On the former plasmid, *malT* is under the control of its own promoter. Upon introduction of pOM25 into strain MC4100rF', carrying plasmid pME1, no toxicity could be observed when IPTG was added to induce expression of MalE251, and no MalE251 protein could be detected in the presence of pOM25 by Western-blot analysis. This was true for both the precursor protein and the mature protein. However, in the presence of pOM25 small amounts of chromosomal

MalE can be seen (Fig. 23).

To demonstrate that pOM25 is responsible for this phenotype, the plasmid was removed from the cells by passaging the cells several times through a medium lacking kanamycin. In the absence of pOM25, the cells exhibited again the lethal phenotype; similarly as in the initial experiments (cf. 3.1, "Results: Construction of the Plasmid encoding MalE251"), the MalE251 polypeptide could be detected with the antibody directed against MalE (Fig. 23). Since in the presence of pOM25 neither the mature MalE251 protein nor its precursor could be visualized in the Western blot, it appears that, in the presence of overproduced MalT, the synthesis of MalE251 was inhibited.

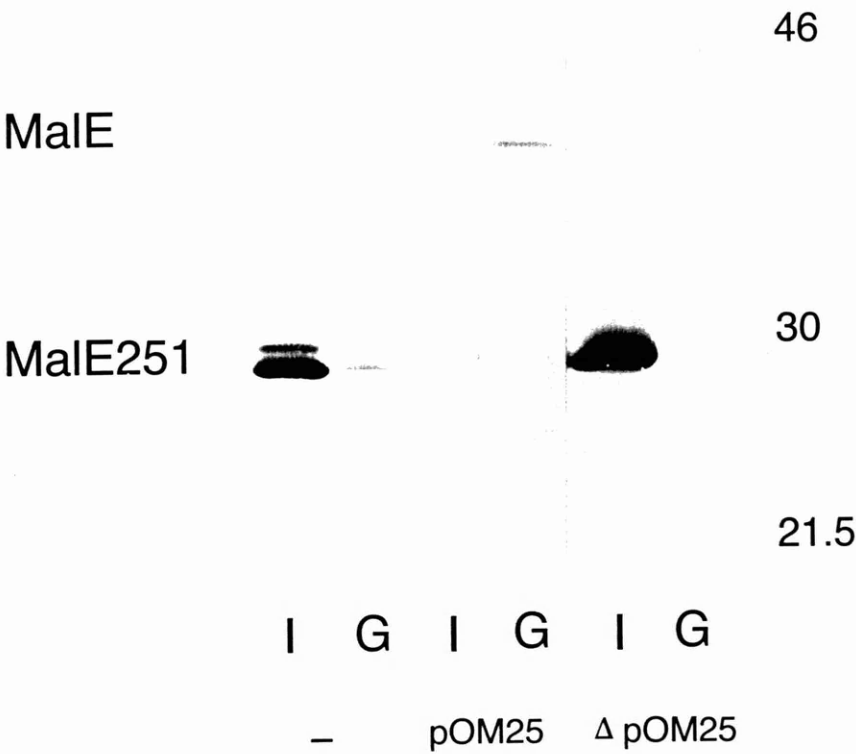


Fig. 23. Western-blot analysis of MalE251. In cells containing plasmid pOM25 (with the gene coding for MalT), in addition to plasmid pME1 (containing the gene coding for MalE251), no MalE251 can be detected with an antibody directed against MalE. When pOM25 is deleted from the cells (Δ pOM25), MalE251 is produced again in massive amounts.

3.9. Regulatory Properties of MalE251

It is well established that for the synthesis of outer membrane proteins, as well as for periplasmic proteins, specific regulatory circuits exist (for review, see Henning and Koebnik, 1994). Therefore, it was rather unexpected that, upon induction of the MalE fragment, several outer membrane proteins, such as OmpA, OmpF, and OmpC appeared to be present at much reduced concentrations (Fig. 10A). It has been demonstrated by Click *et al.* (1988) that overexpression of the outer membrane porin OmpC, for example, leads to a rapid and almost complete block of synthesis of the structurally unrelated OmpA and maltoporin LamB, but has no effect on the production of the periplasmic MalE. Therefore, this phenomenon was quite unexpected, especially since I could demonstrate by pulse chase experiments that the OmpA polypeptide is processed normally, and no accumulation of its precursor occurs (Fig. 14).

4. DISCUSSION

4.1. Toxicity of Hybrid Proteins

Synthesis of the tripartite MalE-OmpA-MalE fusion protein as well as synthesis of the MalE-OmpA hybrid are lethal (Klose *et al.*, 1988). The same is true for the OmpA-MalE hybrid described in this work. Since the OmpA part of these hybrids itself does not cause any toxicity, the question arose whether a combination of both polypeptides is necessary to cause lethality, or whether the MalE fragment alone is responsible for the toxicity observed.

Toxicity caused by some hybrid proteins is a well-known phenomenon, but the mechanisms leading to a lethal phenotype are rather different and often not known at all. Lethality of hybrid proteins has also been reported by Bingle and Smit (1994). The hybrid proteins used in this study were created by fusing alkaline phosphatase or a cellulase reporter to a large *N*-terminal moiety of the *Caulobacter crescentus* surface (S)-layer protein (RsaA). Although the authors were not able to identify the cause of the toxicity of the gene fusions when expressed in *C. crescentus*, they still could show that the toxicity was related to the nature of the hybrid protein. Truncated RsaA peptides lacking their reporter domains were non-toxic. However, the results presented in this thesis demonstrate that the toxicity in the cases of the OmpA-MalE hybrids is not caused by the hybrid protein; rather, the MalE fragment alone caused lethality. In fact, synthesis of the truncated MalE fragment was even more toxic than that of the hybrid protein.

Another example of toxicity caused by a hybrid protein was reported by Silhavy *et al.* (1977). To study protein export in *E. coli*, the authors fused *lacZ* (the gene

specifying β -galactosidase) with the *lamB* gene which codes for the outer membrane protein LamB. High-level synthesis of the hybrid protein results in lethality due to the nature of β -galactosidase: the β -galactosidase portion of the hybrid, being derived from a cytoplasmic protein, contains sequences or structures which are poorly compatible with the export reaction (Lee *et al.*, 1989). Upon induction, proteins that normally are exported (including the hybrid protein) accumulate in the cytoplasm as precursors, and the cells lyse; this phenomenon has been termed "LacZ-hybrid jamming". Overproduction of the SecY inner membrane protein relieves the toxicity caused by the LamB-LacZ hybrid (Bieker and Silhavy, 1989).

Could such a jamming mechanism also be responsible for the toxicity caused by the MalE fragment, as found in the present work? Several lines of evidence indicate that the lethality created by the LamB-LacZ hybrid is the result of a general block in protein export. Considering the fact that synthesis of MalE251 drastically reduced the amounts of other exported proteins such as OmpA, OmpF, and OmpC, this hypothesis appears, at first glance, plausible. However, synthesis of hybrids which cause hybrid jamming lead to the cytoplasmic accumulation of the precursor from many different exported proteins (Bassford *et al.*, 1979; Hall *et al.*, 1982). Using pulse chase experiments, it could be demonstrated that this is not true in the case of MalE251 induction. Synthesis of MalE251 did neither lead to an accumulation of its own precursor, nor to an accumulation of the precursor of another exported protein (e.g., pre-OmpA).

Furthermore, jamming of the export machinery occurs subsequent to signal sequence cleavage, but prior to complete translocation of the entire molecule leaving the hybrid stuck in a transmembrane fashion (Bieker and Silhavy, 1989). Again, this was not true for the MalE hybrids examined in this study. Trypsin digestion, examination

of spheroplasts, and electron microscopic studies demonstrated that the OmpA-MalE hybrid, as well as MalE251, were very efficiently translocated to the periplasm. In conclusion, hybrid jamming cannot be responsible for the toxicity caused by MalE251.

4.2. Selection for Non-Toxic MalE251 Mutants

To obtain more information on why expression of MalE251 exerts high toxicity, a selection for mutants which lacked toxicity was carried out. One problem often encountered in such a selection is to find mutants which are no longer lethal, but which also no longer produce the protein in question. To exclude, as far as possible, such a phenomenon, an operon fusion was constructed, consisting of *malE251* followed by *cat*. Since both genes are controlled by the same promotor (*lac*), synthesis of MalE251 must be accompanied by synthesis of Cat, causing resistance to chloramphenicol of cells producing the MalE fragment. With this method, nine mutants (three of them were plasmid-coded) of independent origin could be selected which were resistant to IPTG, but still produced the MalE fragment.

DNA-sequencing of the three plasmid-coded clones revealed that no mutation had occurred in the *malE251* coding regions in either of the clones. How can the lack of toxicity of these three mutants be explained? As recently stated, the rate of expression of hybrid proteins is one of the parameters determining toxicity (Guigueno *et al.*, 1997). Western-blot analysis of the non-toxic MalE251 mutants showed that the amount of the mature MalE251 produced was much lower than that made by the toxic MalE251 mutant. What could be the reason for such a phenotype?

In order for a plasmid to be maintained stably at a certain copy number per cell, the plasmid must be partitioned so that, upon cell division, each cell receives at least one

copy of the plasmid. Mutants that affect ColE1-related plasmid maintenance could be isolated. It has been reported (Friedman *et al.*, 1984) that mutations in *gyrB* cause instability of pBR322 maintenance in the absence of a selective pressure. Any combination of mutations causing a deficiency of exonuclease I/II and V decreases the stability of maintenance of ColE1-related plasmids (Basset and Kushner, 1984). It is not clear how these mutations act, but they may cause a decrease in the number of the plasmids carrying *malE251* per cell and, therefore, lead to a reduction of the toxicity causing MalE251.

A mutation that decreases the "plasmid copy number" (hence designated *pcnB*) of pBR322 was described by Lopilato *et al.* (1986). The results of this study indicate that *pcnB* mutations with decreased plasmid gene expression are the result of a decreased plasmid copy number. The function of *pcnB* is unknown. It has been suggested that the *pcnB* gene product may be involved in plasmid replication. For example, *pcnB* may encode an endoribonuclease that is required for plasmid primer processing. Furthermore, the authors have suggest that the *pcnB* mutation may alter a subunit of a DNA replication enzyme and, thus, affect plasmid replication.

In summary, the mutants isolated did not help to elucidate the cause of toxicity.

4.3, Loss of Toxicity by the C-Terminally Modified Mutant MalEME2

Deletion of 22 C-terminal residues of MalE251 led to a loss of toxicity. Since the amounts of MalEME2 produced upon induction with IPTG are nearly the same as of MalE251, a gene dosage effect can be excluded as a reason for the relief of toxicity. Why is the C-terminally-deleted clone no longer toxic? Treatment of permeabilized cells with trypsin showed that the mature part of the protein does not become digested, even

when high concentrations (0.5 mg/ml) of the protease were used. This indicates that MalEME2 may not be efficiently secreted to the periplasm. Could a deletion of the 22 C-terminal residues of MalE prevent translocation to the periplasm? Ito and Beckwith (1981) demonstrated that this is not the case. They found that the C-terminal 30 amino acids of MalE are not required for secretion across the cytoplasmic membrane. Betton and Hofnung (1994) could even show that protein fragments of MalE, lacking 65 residues of the C-terminus, are still exported. On the other hand, studies on the secretion of C-terminally-truncated proteins have shown that, although the shortened proteins are translocated based on signal peptide processing, they are not released into the periplasm (Randall *et al.*, 1987). In such a case, however, the protein should be sensitive to degradation by trypsin.

Based on the above considerations, another hypothesis appears to be more likely. It has been reported previously that some MalE mutants with a defective folding of the mature protein in the periplasm lead to the formation of inclusion bodies (Betton and Hofnung, 1996; Betton *et al.*, 1996, Hering *et al.*, 1996). MalE hybrids located in such inclusion bodies are resistant to degradation by *E. coli* proteases (Hering *et al.*, 1996). It is, therefore, likely that MalEME2 cannot be digested by trypsin if it is present in inclusion bodies. Cell fractionation experiments support the latter hypothesis. MalEME2 was entirely recovered in the subcellular insoluble fraction, while no protein could be found in the cytoplasm.

4.4. Suppression of Toxicity Caused by MalE251

In order to better understand the cause of the toxicity exerted by MalE251, the technique of multicopy suppression was used. This method involves selection for genes

which, when overexpressed (i.e., then available “in multicopies”), are able to suppress a certain phenotype. With this approach, a fragment of the recently discovered *nlpE* gene (Snyder *et al.*, 1995) was identified. Overproduction of its gene product, which contains 121 N-terminal residues of NlpE, causes suppression of the toxicity exerted by MalE251. This finding is rather surprising, since NlpE121 misses approximately 50% of the C-terminus of NlpE.

How could overproduction of a lipoprotein fragment cause suppression of the toxicity caused by MalE251? It has been shown by Snyder *et al.* (1995) that increased expression of NlpE suppresses the toxicity of the periplasmic LamB-LacZ-PhoA fusion protein and also of the extracytoplasmic toxicity caused by the mutant outer membrane protein, LamBA23D. In this case, overproduction of NlpE causes suppression of these types of toxicity by activating the Cpx two-component signal transduction pathway.

Work of several laboratories led to the discovery and characterization of this signal transduction pathway. However, the functional role of this system, and the primary signal leading to a cell response, have so far remained elusive. Such systems typically consist of a transmembrane protein which communicates the external signal to a cytoplasmic cognate protein; the latter molecule functions as a transcription factor. This biochemical process involves autophosphorylation of the sensor upon stimulation and is followed by transfer of the phosphoryl group to the regulator (Ronson *et al.*, 1978; Stock *et al.*, 1989; Parkinson and Kofoed, 1992).

The sensor element of the Cpx-operon, *cpxA*, was already identified ten years ago by Weber and Silverman (1988). The CpxA polypeptide is an inner membrane protein consisting of cytoplasmic and periplasmic domains joined by two transmembrane segments. These structural features support that CpxA, like the structurally similar

chemosensory transducers, mediates the response of the cell to environmental variability.

The cognate regulator element of the Cpx two-component signal transduction system, CpxR, was identified only five years after the identification of CpxA. The 232 residue CpxR is homologous to other two-component DNA binding proteins, such as OmpR and ArcA (Dong *et al.*, 1993).

As Danese and co-workers (1995) have shown, the Cpx two-component pathway controls expression of the periplasmic protease DegP, as well as of other factors that are able to combat certain types of extracytoplasmic stress. Overproduction of NlpE acts as a strong signal to induce expression of *degP*. This gene, which is indispensable for bacterial survival at temperatures above 42°C, encodes a major periplasmic protease, DegP (also known as heat shock protein HtrA) (Strauch *et al.*, 1989; Lipinska *et al.*, 1989). Only recently, it was shown that DegP is a peripheral membrane protein which is localized at the periplasmic side of the inner membrane (Skorko-Glonek *et al.*, 1997). The polypeptide is synthesized in form of a 51 kDa precursor protein which is processed to yield a 48 kDa mature protein by removal of a 26-amino acid signal peptide (Strauch *et al.*, 1989; Lipinska *et al.*, 1990).

Mutations in *degP* prevent the degradation of some periplasmic fusion proteins and mutant forms of MalE (Strauch and Beckwith, 1988). It appears that certain stress factors that perturb the cell envelope of *E. coli* increase the synthesis of DegP (Lipinska *et al.*, 1990), whereas the synthesis of cytoplasmic stress proteins has no effect on DegP synthesis (Meccas *et al.*, 1993). This result is consistent with the observation that the *degP* gene is not transcriptionally regulated by the classical heat shock factor σ^{32} . Its transcription is directed by another heat shock factor, σ^B (also referred to as σ^{24} ; Wang

and Kaguni, 1989).

Like σ^{32} , σ^E is thought to direct transcription of a stress regulon (Mecsas *et al.*, 1993). These authors have shown that the activity of σ^E is induced by overproduction of outer membrane proteins, thus suggesting that the signal to which σ^E responds is generated by events occurring in the extracytoplasmic compartments of the cell. This indicates that *E. coli* probably contains two distinct heat-sensitive regulons so that it can mount a distinct response to stress in different compartments of the cell: σ^{32} responds to intracellular stress, while σ^E reacts to stress occurring outside the cytoplasm.

Very little is known about the regulation of σ^E or about its role. A first step towards the characterization of σ^E and its regulon was the recent identification of *rpoE*, the gene encoding σ^E (Rouvière *et al.*, 1995). σ^E controls its own transcription from a σ^E -dependent *rpoE* P2 promoter, indicating that *rpoE* transcription plays a role in the regulation of σ^E activity in the cell. First results demonstrate that the σ^E regulon comprises, at least, eleven members including DegP, σ^{32} , and σ^E itself (Rouvière *et al.*, 1995).

In addition to the regulation of *degP* transcription, the σ^E regulon is also involved in maintaining the integrity of the outer membrane or the cell envelope. Even at normal temperature (30°C), $\Delta rpoE$ cells have an increased sensitivity to chemicals known to disrupt the outer membrane (such as 1 mM EDTA) and to cause an increased permeability to hydrophobic antibiotics, such as rifampicin. This strongly suggests that $\Delta rpoE$ cells have a defective envelope.

Protein misfolding in the cell envelope, as well as other stress factors to the bacterial extracytoplasmic medium, are known to increase synthesis of DegP. Since the degradation of abnormal proteins is normally performed by DegP, why is MalE251 not

digested by this protease without the induction of the Cpx-pathway induced by the overproduction of NlpE/NlpE121?

One possibility is that induction of DegP synthesis by MalE251 is not strong enough to produce amounts of the protease sufficient to degrade the MalE fragment. It is known that the *degP* gene is heat inducible (Lipinska *et al.*, 1989). Therefore, I tried to reduce the toxicity caused by MalE251 by increasing the temperature during growth to up to 45°C. However, neither at 43°C, 44°C, or 45°C was a reduction of the toxicity observed. At higher temperatures, the induced cells died even more rapidly than the cells grown without IPTG. Hence, induction of *degP* by this mechanism is insufficient to combat the toxicity caused by MalE251. Similar results have been reported by Snyder *et al.* (1995). These authors made use of the fact that overproduction of outer membrane proteins increases *degP* synthesis. However, *degP* induction by overexpression of *ompF* did not suppress the toxicity caused by the LamB-LacZ-PhoA fusion proteins (Snyder *et al.*, 1995).

It is difficult to understand how induction of *degP* via the Cpx-pathway is able to suppress the toxicities caused by LamB-LacZ-PhoA or MalE251, while induction of *degP* by elevated temperatures or by overproduction of *ompF* is not able to cause suppression. The system is complicated, because *degP* is controlled by at least two different signalling pathways: the Cpx-pathway and the Eo^E-dependent regulon.

The latter pathway leads to another possible explanation why DegP in cells without high levels of NlpE or NlpE121 is not able to digest MalE251 sufficiently. Previous work has demonstrated that *degP* transcription is modulated in response to the amount of outer membrane proteins by modulating the activity of the heat shock factor Eo^E (Mecsas *et al.*, 1993; Raina *et al.*, 1995; Rouvière *et al.*, 1995). In particular, high-

level synthesis of the outer membrane porins OmpF and OmpC increased the activity of Eo^E. On the other hand, mutations that decrease the number of proteins located in the outer membrane decrease the activity of Eo^E. As described above (cf. 3.9, "Results: Regulatory Properties of MalE251"), synthesis of MalE251 appeared to lead to a drastic reduction in the amounts of several outer membrane proteins (e.g., OmpA, OmpC, OmpF). Hence, such a decrease in the number of outer membrane proteins might lead to a decrease in the synthesis of DegP to a level which is no longer sufficient to digest MalE251 efficiently.

Although the results presented by Snyder *et al.* (1995), as well as those described in the present work, strongly indicate that high levels of NlpE or NlpE121 activate *degP* transcription via the Cpx-pathway, one cannot rule out the following possibility: expression of very high levels of NlpE may lead to misfolded fractions of NlpE itself which may not be able to assemble correctly into the outer membrane. Indeed, fractionation experiments (Snyder *et al.*, 1995) showed that overproduction of NlpE leads to several degradation products of NlpE. Misfolding of the protein itself can be especially envisaged in the case of NlpE121 which misses approximately 50 % of the C-terminus missing. Does this mean that CpxA recognizes overexpressed NlpE/NlpE121 as "junk"? This is unlikely, since it has been shown that overexpression of other outer membrane proteins, even other lipoproteins, does not cause Cpx-mediated *degP* induction, nor does it cause suppression of toxicity (Danese *et al.*, 1995).

It is possible, however, that, in addition to its interaction with CpxA, high levels of NlpE or NlpE121 may lead to a response of the Eo^E signalling pathway responding to protein misfolding in the cell envelope. It was found recently (Bass *et al.*, 1996) that the truncated rare lipoprotein A (RlpA), which lacks 243 C-terminal residues of the 345

residue mature RlpA, is able to suppress a mutant *prc* in *E. coli*. Strains with a *prc* null mutation exhibit a conditional lethal phenotype. Cells die at temperatures of 40°C or higher if grown in hypotonic media (Hara *et al.*, 1991). The mechanism of Prc suppression by the truncated RlpA is unclear. Since it is unlikely that a protein with a deletion of 70% is able to function normally, the authors suggest that it may indirectly lead to the induction of the DegP protease via induction of the σ^E heat shock factor.

4.5. Reasons for the Toxicity Caused by Expression of MalE251

It has been shown in this study that the MalE fragment alone is responsible for the lethal phenotype. It could be excluded that MalE251 causes jamming of the export machinery as was the case for some LamB-LacZ hybrids (Silhavy *et al.*, 1977). By analysing spheroplasts, it could be demonstrated that MalE251 is released from the plasma membrane. An impairment of the release mechanism is known from the C-terminally truncated periplasmic phosphodiesterase (Hengge and Boos, 1985; Hengge-Aronis and Boos, 1986). This protein is translocated across the plasma membrane, but not released into the periplasm. Klose *et al.* (1988) hypothesized that this is the cause for the toxicity observed with two different hybrid proteins that contain a fragment of MalE consisting of the area between residues 120 and 370.

If all these mechanisms do not apply for toxicity of MalE251, what causes the lethal phenotype? There are several lines of evidence pointing to the possibility that the cell envelope of cells expressing MalE251 is not intact. Firstly, MalE251 expressing cells lyse even in an osmotically protected environment. Secondly, synthesis of MalE251 causes the expression of the recently discovered Spy protein (S. Hagenmaier, personal communication). The periplasmic Spy protein is normally not detectable at all,

even under environmental stress conditions, such as high temperatures, high salt concentrations, etc., but is produced only in spheroplasts (Hagenmaier *et al.*, 1997). Synthesis of MalE251, therefore, seems to present a similar stress situation for the cell as does spheroplast formation.

Very recently, a new member of the Cpx regulon, CpxP, was found (Danese and Silhavy, 1998). CpxP is a periplasmic protein whose synthesis is increased at the transcriptional level by the Cpx system. Like DegP, CpxP is utilized by the activated Cpx-pathway to suppress toxicity caused by exported fusion proteins, such as LamB-LacZX90 (Danese and Silhavy, 1998). Most interestingly, CpxP and Spy exhibit a certain degree of identity (29% over 101 amino acids). This might be another indication that the expression of Spy, caused by the synthesis of MalE251, is a response to the extracytoplasmic stress evoked by MalE251.

A defect in the cell envelope may also be explained by the fact that, upon synthesis of MalE251, the amounts of several outer membrane proteins appeared to be reduced. A reduction of synthesis of those outer membrane proteins might easily lead to a destabilisation of the outer membrane. The phenomenon of the down-regulation of outer membrane proteins was rather unexpected. It has been demonstrated previously that overexpression of the outer membrane protein OmpC lead to an almost complete block of synthesis of OmpA and LamB, while it has no effect on the production of the periplasmic MalE (Click *et al.*, 1988). The specificity of such feedback mechanisms is also supported by the results of Ried *et al.* (1990). The authors showed that synthesis of several altered OmpA proteins caused a decrease of the cellular concentration of the porins OmpC and OmpF, but had no effect on the synthesis of the periplasmic protein β -lactamase. A similar type of specificity has been reported for periplasmic proteins

(Hengge and Boos, 1985; Hengge-Aronis and Boos, 1986).

Since the results of the present work are in contradiction to the hypothesis that distinct mechanisms exist that regulate the synthesis of outer membrane proteins and periplasmic proteins, further experiments on the regulatory properties of MalE251 would be necessary to clarify this issue. It cannot be excluded that the total amount of protein used for SDS-PAGE representing the IPTG-induced cells was actually lower than it was in the case of the non-induced cells. Consequently, also the amount of porins, as well as that of OmpA, would appear to be lower in the induced cells than in the non-induced cells.

To examine whether synthesis of malE251 does, indeed, have a regulatory effect on the synthesis of outer membrane proteins, quantitative autoradiography could be carried out. This would include pulse-labelling of cultures with [³⁵S]methionine, followed by chase with unlabelled methionine after various times of IPTG induction. It would also be important to include a zero-time point before the addition of IPTG. Following immunoprecipitation with antibodies directed against MalE, OmpA, OmpC, and OmpF, scanning densitometry would be performed.

Another possible cause for the defective cell envelope of the MalE251 mutants could be a titration by MalE251 of one or several components necessary for cell envelope formation. The genes for at least seven periplasmic proteases of *E. coli* have been cloned, and there is evidence that more exist. In order to identify new protease genes, Bass *et al.* (1996) isolated multicopy suppressors of the conditional lethal phenotype exhibited by a *prc* null strain grown at high temperature in hypotonic medium. Prc has been shown *in vivo* to be a protease that cleaves the C-terminus of FtsI (penicillin-binding protein 3) (Nagasawa *et al.*, 1989). Although FtsI is not an excreted

protein, it is synthesized as a precursor form and then processed to a mature form (Nakamura *et al.*, 1983). Interestingly, processing of the precursor protein does not involve *N*-terminal cleavage; rather, the *C*-terminal part is processed. FtsI is an essential enzyme required for septum formation in the dividing cell (Spratt, 1977). It catalyses the transglycosylation and transpeptidation reactions in the final steps of peptidoglycan synthesis (Ishino and Matsushashi, 1981).

Four suppressors of the conditional lethal phenotype of the *prc* null mutant could be isolated: Two DegP protease homologues HhoA and HhoB, as well as DksA and a truncated RlpA. The finding that multicopy suppressors of the *prc* mutant include two new members of the DegP family suggests that these have overlapping substrate specificities (Bass *et al.*, 1996). Prc may combine with members of the DegP protein family to help refold or degrade misfolded proteins in the periplasm. If an involvement of Prc in the degradation of MalE251 occurred, one could imagine that other functions of Prc might be compromised.

4.6. The Cpx Two-Component Signal Transduction Pathway:

Signalling, Regulation, and Response

It is well established that the σ^E regulon of *E. coli* is induced by misfolded or unstable proteins located in the cell envelope. *degP*, a member of this regulon, encodes the periplasmic protease DegP, which is involved in the degradation of misfolded proteins in the extracytoplasm. Interestingly, *degP* transcription is also under positive control of the CpxA/CpxR two-component signal transduction system (Raina *et al.*, 1995; Danese *et al.*, 1995). Activation of the Cpx pathway, however, does not only result in elevated expression of DegP, but also of the periplasmic enzyme DsbA (Danese

and Silhavy, 1997), the peptidyl-propyl isomerase PpiA (Pogliano *et al.*, 1997), and the periplasmic protein CpxP (Danese and Silhavy, 1998a). While transcription of *degP* is coregulated by both σ^E and CpxA/R, *dsbA*, *ppiA* and *cpxP* are not affected by alterations in σ^E activity.

In recent years, several laboratories have elucidated, in great detail, the mechanisms underlying signal transduction in response to envelope stress. In order to gain a better understanding of how signalling for increased transcription of *degP* is transduced between the cell envelope and the cytoplasm, Missiakas and Raina (1997) performed extensive mutagenesis experiments. By using this approach, the authors were able to isolate three loci which affected *degP* transcription. The mutations could be mapped to the *cpxA/cpxR* operon and the two newly identified genes *prpA* and *prpB*. PrpA and PrpB are about 50% identical at the amino acid level. Based on sequence homology, especially in their catalytic domains, they appear to be prototypes of classical eukaryotic type I serine/threonine phosphatases. Both Prp proteins are able to dephosphorylate *in vitro* proteins which are phosphorylated on serine, threonine, and tyrosine residues. Moreover, their *in vivo* interaction with the Cpx two-component system suggests that they are also able to act as aspartate and/or histidine phosphatases (Missiakas and Raina, 1997).

Further, it was shown by the latter authors that the phosphatase activity of PrpA, and also to some extent of PrpB, plays an active part in the induction of *degP* transcription. Overexpression of PrpA and PrpB leads to an approximately fivefold increase in *degP* transcription. Moreover, some chromosomal point mutations which map to the *prpA* and *prpB* genes result in a decrease in *degP* transcription. When multiple copies of *prpA* and *prpB* are present, these genes were also found in complementary

experiments to be able to induce transcription from *degP*, even in the absence of misfolded proteins. This effect, which is best demonstrated by overproducing PrpA, is dependent on the presence of a wild-type functioning copy of CpxR and CpxA. Thus, PrpA and PrpB act as positive modulators of *cpx*-dependent activation of *degP* transcription. The Prp phosphatases also appear to modulate the activity of other two-component systems besides Cpx and may have a more general role in protein phosphorylation (Missiakas and Raina, 1997).

There are several lines of evidence supporting the hypothesis that the Cpx two-component system monitors and regulates the physiology of extracytoplasmic proteins. However, the primary stimulus leading to a cell response is still not fully known. The Cpx system is activated by overproduction of certain extracytoplasmic proteins. This could be demonstrated for the outer membrane lipoprotein NlpE (Snyder *et al.*, 1995), the NlpE fragment NlpE121 (this thesis), and for the P-pili subunits PapE and PapG (Jones *et al.*, 1997). While the function of NlpE is not yet known, Pap G and PapE are surface appendages synthesized by uropathogenic strains of *E. coli* (Jones *et al.*, 1997). Stimulation of the Cpx pathway by overproduction of either PapG or PapE suggests that CpxA may monitor the assembly of extracytoplasmic protein structures, such as pili (Danese and Silhavy, 1998b).

In addition to protein-mediated stimuli, CpxA gets activated by null mutations in *dsbD*. This gene encodes the oxidoreductase DsbD, which is required for proper disulfide bond formation in periplasmic proteins. Induction of *degP* transcription in a strain which is deficient in the periplasmic folding catalyst DsbD is fully optimised only, if, in addition to CpxA, functional CpxR and PrpA proteins are present (Missiakas and Raina, 1997).

Recently, it was reported that substitution of the major phospholipid of the *E. coli* cell envelope phosphatidylethanolamine by the normal anionic phospholipids phosphatidylglycerol and cardiolipin results in constitutive activation of the Cpx two-component signal transduction pathway (Mileykovskaya and Dowhan, 1997). The authors of this study propose that alteration in phospholipid composition of the inner membrane of *E. coli* results in conformational changes in the CpxA protein. These changes mimic the alterations caused by point mutations in CpxA, thereby eliciting a gain-of-function phenotype in this locus. So far, the role of phospholipids in bacterial signal transduction has not been studied directly. However, several investigations carried out on eukaryotic signal transduction systems point to an important role in protein-lipid interactions. The phospholipid environment with its polar head group and acyl chain composition has been shown to affect the structural and functional properties of membrane-bound receptors. This phenomenon has been demonstrated for rhodopsin (Gibson and Brown, 1993), the insulin receptor (McCallum and Epand, 1995), and a receptor loop of the protein expressed by the *mas* oncogene (Pertinhez *et al.*, 1995).

The Cpx system can also be activated by alkaline pH (Danese and Silhavy, 1998a). These authors have demonstrated that transcription of *cpxP* is strongly induced by alkaline pH in a CpxA-dependant manner. Although the mechanism by which alkaline conditions activate CpxA is not known, it is possible that high pH perturbs extracytoplasmic protein physiology (Danese and Silhavy, 1998b).

Based on the above results and the characterisation of gain-of-function Cpx* mutations, Raivio and Silhavy (1997) proposed the following working model for Cpx-mediated signal transduction: CpxA has three enzymatic activities (autokinase, kinase, and phosphatase) through which it controls the levels of CpxR-P. Phosphorylation of

CpxR confers increased affinity for the promoters of the Cpx regulon members, thus leading to transcriptional activation and elevated expression. The output of the regulon is controlled through modulations in the CpxA kinase/phosphatase activity ratio in response to the conditions in the cell envelope. Under normal physiological conditions, a putative periplasmic ligand binds to the periplasmic domain of CpxA, maintaining CpxA and the Cpx regulon in a down-regulated state. Envelope stress results in loss of interaction with the effector and, thus, in activation of the regulon.

4.7. Perspectives

In the present thesis, it has been demonstrated that overproduction of the NlpE-fragment NlpE121 eliminates the toxicity caused by synthesis of MalE251. Although the results strongly indicate that high levels of NlpE121 activate *degP* transcription via the Cpx-pathway, it cannot be ruled out that high expression of NlpE121 could lead to misfolded fractions of the protein fragment itself, resulting in a response of the previously described σ^E regulon. To elucidate the mechanisms further by which NlpE121 eliminates the MalE251-caused toxicity, additional experiments are required. They will be outlined in the following.

In 1995, Danese and co-workers pointed out that the NlpE sequence contains a serine protease motif, suggesting that it may interact with serine proteases, such as DegP. At its C-terminus, NlpE121 also contains the serine protease motif. C-terminal truncation of NlpE121 is likely to elucidate whether this motif also plays a functional role for the suppression of MalE251-caused toxicity. Furthermore, C-terminal deletions could answer the question whether or not NlpE121 is recognized as a misfolded protein. Alternatively, the partial NlpE protein may be sufficient to cause a distinct DegP

response via the Cpx two-component pathway. By means of ExoIII nuclease, the C-terminal end of NlpE121 could be stepwise deleted and the newly obtained fragments transformed into strain MC4100rF' which carries pME1-cat.

Induction with IPTG is likely to reveal whether the C-terminal fragments of NlpE121 are still able to suppress the toxicity caused by synthesis of MalE251. Lack of suppression would indicate that (1) the Cpx pathway is, indeed, activated by NlpE121, and (2) the serine protease motif is likely to be involved in Cpx-mediated suppression. Suppression of MalE251-caused toxicity by one of the C-terminally shortened clones, on the other hand, would prompt sequencing of the gene which encodes the protein fragment.

The latter result would, however, leave the question unanswered whether or not this new NlpE fragment, as well as NlpE121, are operating via the Cpx pathway. Therefore, further experiments would have to be carried out. If the misfolded state of NlpE121 were, indeed, the cause for σ^E -controlled synthesis of DegP, then NlpE121 itself should also be a target for proteolytic degradation by DegP. Therefore, pulse-chase experiments using [³⁵S]methionine, followed by immunoprecipitation with an antibody directed against NlpE, could be performed. This experiment is likely to reveal not only the fate of NlpE121, but also whether suppression of MalE251-caused toxicity is eliminated via the Cpx pathway.

Finally, it would be interesting to determine the levels of DegP under different conditions. For this set of experiments, Western blot analysis, employing an antibody against DegP, could be performed. In the first subset of experiments, cells with only one plasmid carrying *malE251* would be grown. In the second subset of experiments, two plasmids, one carrying *malE251*, the other *nlpE121*, would be used. In the third

subset of experiments, *nlpE121* would be exchanged by *nlpE*. It would then be interesting to see whether NlpE121 is as useful as an inducer of *degP* as it is when using the whole NlpE protein. These experiments might help to elucidate further the mechanisms underlying the Cpx two-component signal transduction system.

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6. REFERENCES

- Albin, R. and Silverman, P. M. (1984). Physical and genetic structure of the *glpK-cpxA* interval of the *Escherichia coli* K-12 chromosome. *Mol. Gen. Genet.* **197**, 261-271.
- Ames, G. F.-L. (1986). Bacterial periplasmic transport systems: structure, mechanism, and evolution. *Annu. Rev. Biochem.* **55**, 397-425.
- Ames, G. F.-L. and Nikaido, K. (1976). Two-dimensional gel electrophoresis of membrane proteins. *Biochemistry* **15**, 616-623.
- Bass, S., Gu, Q. and Christen, A. (1996). Multicopy suppressors of Prc mutant *Escherichia coli* include two HtrA (DegP) protease homologs (HhoAB), DksA, and a truncated RlpA. *J. Bacteriol.* **178**, 1154-1161.
- Bassett, C. L. and Kushner, S. R. (1984). Exonucleases I, III, and V are required for stability of ColE1-related plasmids in *Escherichia coli*. *J. Bacteriol.* **157**, 661-664.
- Bassford, P. J., Jr., Silhavy, T. J. and Beckwith, J. R. (1979). Use of gene fusion to study secretion of maltose-binding protein into *Escherichia coli* periplasm. *J. Bacteriol.* **139**, 19-31.
- Bavoil, P. and Nikaido, H. (1981). Physical Interaction between the phage λ receptor protein and the carrier-immobilized maltose-binding protein of *Escherichia coli*. *J. Biol. Chem.* **256**, 11385-11388.
- Bavoil, P., Wandersman, C., Schwartz, M. and Nikaido, H. (1983). A mutant form of maltose-binding protein of *Escherichia coli* deficient in its interaction with the bacteriophage lambda receptor protein. *J. Bacteriol.* **155**, 919-921.
- Bayer, M. E. (1968). Areas of adhesion between wall and membrane of *Escherichia coli*. *J. Gen. Microbiol.* **53**, 395-404.
- Bayer, M. E. (1991). Zones of membrane adhesion in the cryofixed envelope of *Escherichia coli*. *J. Struct. Biol.* **107**, 268-280.
- Bedouelle, H., Bassford, P. J., Fowler, A. V., Zabin, I., Beckwith, J. and Hofnung, M. (1980). Mutations which alter the function of the signal sequence of the maltose binding protein of *Escherichia coli*. *Nature* **285**, 78-81.

- Betton, J.-M., Boscus, D., Missiakas, D., Raina, S. and Hofnung, M. (1996). Probing the structural role of an $\alpha\beta$ loop of maltose-binding protein by mutagenesis: heat-shock induction by loop variants of the maltose-binding protein that form periplasmic inclusion bodies. *J. Mol. Biol.* **262**, 140-150.
- Betton, J.-M. and Hofnung, M. (1994). *In vivo* assembly of active maltose binding protein from independently exported protein fragments. *EMBO J.* **13**, 1226-1234.
- Betton, J.-M. and Hofnung, M. (1996). Folding of a mutant maltose-binding protein of *Escherichia coli* which forms inclusion bodies. *J. Biol. Chem.* **271**, 8046-8052.
- Bieker, K. and Silhavy, T. J. (1989). PrlA is important for the translocation of exported proteins across the cytoplasmic membrane of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **86**, 968-972.
- Bieker-Brady, K. and Silhavy, T. J. (1992). Suppressor analysis suggests a multistep, cyclic mechanism for protein secretion in *Escherichia coli*. *EMBO J.* **11**, 3165-3174.
- Bingle, W. H. and Smit, J. (1994). Alkaline phosphatase and a cellulase reporter protein are not exported from the cytoplasm when fused to large N-terminal portions of the *Caulobacter crescentus* surface (S)-layer protein. *Can. J. Microbiol.* **40**, 777-782.
- Blattner, F. R., Plunkett III, G., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B. and Ying, S. (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453-1462.
- Boos, W. and Staehelin, A. L. (1981). Ultrastructural localization of the maltose-binding protein within the cell envelope of *Escherichia coli*. *Arch. Microbiol.* **129**, 240-246.
- Braun, V. (1975). Covalent lipoprotein from the outer membrane of *Escherichia coli*. *Biochem. Biophys. Acta* **415**, 335-377.
- Bremer, E., Beck, E., Hindennach, I., Sonntag, I. and Henning, U. (1980). Cloned structural gene (*ompA*) for an integral outer membrane protein of *Escherichia coli* K-12. *Mol. Gen. Genet.* **179**, 13-20.
- Brundage, L., Fimmel, C. J., Mizushima, S. and Wickner, W. (1992). SecY, SecE, and band 1 form the membrane-embedded domain of *Escherichia coli* preprotein translocase. *J. Biol. Chem.* **267**, 4166-4170.

- Brundage, L., Hendrick, J. P., Schiebel, E., Driessen, A. J. M. and Wickner, W. (1990). The purified *E. coli* integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. *Cell* **62**, 649-657.
- Cabelli, R. J., Dolan, K. M., Qian, L. and Oliver, D. B. (1991). Characterization of membrane-associated and soluble states of SecA protein from wild-type and SecA51 (TS) mutant strains of *Escherichia coli*. *J. Biol. Chem.* **266**, 24420-24427.
- Carlemalm, E., Garavito, R. M. and Villiger, W. (1982). Resin development for electron microscopy and an analysis of embedding at low temperature. *J. Microsc.* **126**, 123-143.
- Carlson, J. H. and Silhavy, T. J. (1993). Signal sequence processing is required for the assembly of Lamb trimers in the outer membrane of *Escherichia coli*. *J. Bacteriol.* **175**, 3327-3334.
- Casadaban, M. J. (1976). Transposition and fusion of the *lac* genes to select genes in *Escherichia coli* using bacteriophage Lambda and Mu. *J. Mol. Biol.* **104**, 541-553.
- Chen, R. and Henning, U. (1996). A periplasmic protein (SkP) of *Escherichia coli* selectively binds a class of outer membrane proteins. *Mol. Microbiol.* **19**, 1287-1294.
- Chen, R., Schmidmayr, W., Krämer, C., Chen-Schmeisser, U. and Henning, U. (1980). Primary structure of major outer membrane protein Π^* (*ompA* protein) of *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. USA* **77**, 4592-4596.
- Chung, C. T., Niemela, S. L. and Miller, R. H. (1989). One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. USA* **86**, 2172-2175.
- Click, E. M., McDonald, G. A. and Schnaitman, C. A. (1988). Translational control of exported proteins that results from OmpC porin overexpression. *J. Bacteriol.* **170**, 2005-2011.
- Cohen, S. N., Chang, A. C. Y. and Hsu, L. (1972). Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. USA* **69**, 2110-2114.
- Cole, S. T. and Raibaud, O. (1986). The nucleotide sequence of the *malT* gene encoding the positive regulator of the *Escherichia coli* maltose regulon. *Gene* **42**, 201-208.
- Cosma, C. L., Danese, P. N., Carlson, J. H., Silhavy, T. J. and Snyder, W. B. (1995). Mutational activation of the Cpx signal transduction pathway of *Escherichia coli* suppresses the toxicity

- conferred by certain envelope-associated stresses. *Mol. Microbiol.* **18**, 491-505.
- Cunningham, K. and Wickner, W. (1989). Specific recognition of the leader region of precursor proteins is required for the activation of translocation ATPase of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **86**, 8630-8634.
- Danese, P. N. and Silhavy, T. J. (1997). The σ^E and the Cpx signal transduction systems control the synthesis of periplasmic protein-folding enzymes in *Escherichia coli*. *Genes Dev.* **11**, 1183-1193.
- Danese, P. N. and Silhavy, T. J. (1998a). CpxP, a stress-combative member of the Cpx regulon. *J. Bacteriol.* **180**, 831-839.
- Danese, P. N. and Silhavy, T. J. (1998b). Targeting and assembly of periplasmic and outer-membrane proteins in *Escherichia coli*. *Annu. Rev. Genet.* **32**, 59-94.
- Danese, P. N., Snyder, W. B., Cosma, C. L., Davis, L. J. B. and Silhavy, T. J. (1995). The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP. *Genes & Dev.* **9**, 387-398.
- Datta, D. B., Arden, B. and Henning, U. (1977). Major proteins of the *Escherichia coli* outer cell envelope membrane as bacteriophage receptors. *J. Bacteriol.* **131**, 821-829.
- Dierstein, R. and Wickner, W. (1985). The leader region of pre-maltose binding protein binds amphiphiles. *J. Biol. Chem.* **260**, 15919-15924.
- Dong, J., Iuchi, S., Kwan, H.-S., Lu, Z. and Lin, E. C. C. (1993). The deduced amino-acid sequence of the cloned *cpxR* gene suggests the protein is the cognate regulator for the membrane sensor, CpxA, in a two-component signal transduction system of *Escherichia coli*. *Gene* **136**, 227-230.
- Duplay, P., Bedouelle, H., Fowler, A., Zabin, I., Saurin, W. and Hofnung, M. (1984). Sequences of the *malE* gene and of its product, the maltose-binding protein of *Escherichia coli* K12. *J. Biol. Chem.* **259**, 10606-10613.
- Emr, S. D., Hanley-Way, S. and Silhavy, T. J. (1981). Suppressor mutations that restore export of a protein with a defective signal sequence. *Cell* **23**, 79-88.
- Eppens, E. F., Nouwen, N. and Tommassen, J. (1997). Folding of a bacterial outer membrane protein during passage through the periplasm. *EMBO J.* **16**, 4295-4301.
- Fikes, J. D. and Bassford, P. J. (1989). Novel *secA* alleles improve export of maltose-binding protein

- synthesized with a defective signal peptide. *J. Bacteriol.* **171**, 402-409.
- Freudl, R., Schwarz, H., Klose, M., Movva, N. R. and Henning, U. (1985). The nature of information, required for export and sorting, present within the outer membrane protein OmpA of *Escherichia coli* K-12. *EMBO J.* **4**, 3593-3598.
- Friedman, D. I., Plantefaber, L. C., Olson, E. J., Carver, D., M.H., O. and Gellert, M. (1984). Mutations in the DNA *gyrB* gene that are temperature sensitive for lambda site-specific recombination, mu growth, and plasmid maintenance. *J. Bacteriol.* **157**, 490-497.
- Gardel, C., Benson, S., Hunt, J., Michaelis, S. and Beckwith, J. (1987). *secD*, a new gene involved in protein export in *Escherichia coli*. *J. Bacteriol.* **169**, 1286-1290.
- Gardel, C., Johnson, K., Jacq, A. and Beckwith, J. (1990). The *secD* locus of *E. coli* codes for two membrane proteins required for protein export. *EMBO J.* **9**, 3209-3216.
- Ghrayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y. and Inouye, M. (1984). Secretion vectors in *Escherichia coli*. *EMBO J.* **3**, 2437-2442.
- Gibson, N. J. and Brown, M. F. (1993). Lipid headgroup and acyl chain composition modulate the MI-MII equilibrium of rhodopsin in recombinant membranes. *Biochemistry* **32**, 2438-2454.
- Guigueno, A., Belin, P. and Boquet, P. L. (1997). Defective export in *Escherichia coli* caused by DsbA'-PhoA hybrid proteins whose DsbA' domain cannot fold into a conformation resistant to periplasmic proteases. *J. Bacteriol.* **179**, 3260-3269.
- Gupta, S. D., Lee, B. T. O., Camakaris, J. and Wu, H. C. (1995). Identification of *cutC* and *cutF* (*nlpE*) genes involved in copper tolerance in *Escherichia coli*. *J. Bacteriol.* **177**, 4207-4215.
- Hagenmaier, S., Stierhof, Y. D. and Henning, U. (1997). A new periplasmic protein of *Escherichia coli* which is synthesized in spheroplasts but not in intact cells. *J. Bacteriol.* **179**, 2073-2076.
- Hall, M. N., Schwartz, M. and Silhavy, T. J. (1982). Sequence information within the *lamB* gene is required for proper routing of the bacteriophage λ receptor protein to the outer membrane of *Escherichia coli* K-12. *J. Mol. Biol.* **156**, 93-112.
- Hartl, F.-U., Lecker, S., Schiebel, E., Hendrick, J. P. and Wickner, W. (1990). The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the *E. coli* plasma membrane. *Cell* **63**, 269-279.
- Hayashi, H. and Ohba, M. (1982). Immobilization of the periplasmic maltose-binding protein of

Escherichia coli. *Ann. Microbiol. (Paris)* **133A**, 195-197.

Hendrick, J. P. and Wickner, W. (1991). SecA protein needs both acidic phospholipids and SecY/E protein for functional high-affinity binding to the *Escherichia coli* plasma membrane. *J. Biol. Chem.* **266**, 24596-24600.

Hengge, R. and Boos, W. (1985). Defective secretion of maltose and ribose-binding proteins caused by a truncated periplasmic protein in *Escherichia coli*. *J. Bacteriol.* **162**, 972-978.

Hengge-Aronis, R. and Boos, W. (1986). Translational control of exported proteins in *Escherichia coli*. *J. Bacteriol.* **167**, 462-466.

Henning, U. and Koebnik, R. (1994). Outer membrane proteins of *Escherichia coli*: mechanism of sorting and regulation of synthesis. In *Bacterial Cell Wall* (ed. J.-M. Ghuysen and R. Hakenbeck), pp. 381-394. Elsevier Science Publishers.

Henning, U., Royer, H.-D., Teather, R. M., Hindennach, I. and Hollenberg, C. P. (1979). Cloning of the structural gene (*ompA*) for an integral outer membrane protein of *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. USA* **76**, 4360-4364.

Hering, T. M., Kollar, J., Huynh, T. D. and Varelas, J. B. (1996). Purification and characterization of decorin core protein expressed in *Escherichia coli* as a maltose-binding protein fusion. *Anal. Biochem.* **240**, 98-108.

Heuzenroeder, M. W. and Reeves, P. (1980). Periplasmic maltose-binding protein confers specificity on the outer membrane maltose pore of *Escherichia coli*. *J. Bacteriol.* **141**, 431-435.

Hiratsu, K., Amemura, M., Nashimoto, H., Shinagawa, H. and Makino, K. (1995). The *rpoE* gene of *Escherichia coli*, which encodes σ^E , is essential for bacterial growth at high temperature. *J. Bacteriol.* **177**, 2918-2922.

Hobot, J. A., Carlemalm, E., Villiger, W. and Kellenberger, E. (1984). Periplasmic gel: new concept resulting from the reinvestigation of bacterial cell envelope ultrastructure by new methods. *J. Bacteriol.* **160**, 143-152.

Inouye, M., Shaw, J. and Shen, C. (1972). The assembly of a structural lipoprotein in the envelope of *Escherichia coli*. *J. Biol. Chem.* **247**, 8154-8159.

Ishino, F. and Matsushashi, M. (1981). Peptidoglycan synthetic enzyme activities of highly purified penicillin-binding protein 3 in *Escherichia coli*: a septum-forming reaction sequence. *Biochem.*

Biophys. Res. Commun. **101**, 905-911.

Ito, K. and Beckwith, J. R. (1981). Role of the mature protein sequence of maltose-binding protein in its secretion across the *E. coli* cytoplasmic membrane. *Cell* **25**, 143-150.

Jap, B. K. and Walian, P. J. (1990). Biophysics of the structure and function of porins. *Quart. Rev. Biophysics* **23**, 367-403.

Jobling, M. G. and Holmes, R. K. (1990). Construction of vectors with the p15a replicon, kanamycin resistance, inducible *lacZ α* and pUC18 or pUC19 multiple cloning sites. *Nucl. Acids Res.* **18**, 5315-5316.

Jones, C. H., Danese, P. N., Pinkner, J. S., Silhavy, T. J. and Hultgren, S. J. (1997). The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction systems. *EMBO J.* **16**, 6394-6406.

Kellenberger, E. (1990). The 'Bayer bridges' confronted with results from improved electron microscopy methods. *Mol. Microbiol.* **4**, 697-705.

Klose, M., MacIntyre, S., Schwarz, H. and Henning, U. (1988b). The influence of amino substitutions within the mature part of an *Escherichia coli* outer membrane protein (OmpA) on assembly of the polypeptide into its membrane. *J. Biol. Chem.* **263**, 13297-13302.

Klose, M., Schwarz, H., MacIntyre, S., Freudl, R., Eschbach, M.-L. and Henning, U. (1988a). Internal deletions in the gene for an *Escherichia coli* outer membrane protein define an area possibly important for recognition of the outer membrane by this polypeptide. *J. Biol. Chem.* **263**, 13291-13296.

Koiwai, O. and Hayashi, H. (1979). Studies on bacterial chemotaxis. *J. Biochem.* **86**, 27-34.

Krikos, A., Mutoh, N., Boyd, A. and Simon, M. I. (1983). Sensory transducers of *E. coli* are composed of discrete structural and functional domains. *Cell* **33**, 615-622.

Kumamoto, C. A. (1991). Molecular chaperones and protein translocation across the *Escherichia coli* inner membrane. *Mol. Microbiol.* **5**, 19-22.

Kumamoto, C. A., Chen, L., Fandl, J. and Tai, P. C. (1989). Purification of the *Escherichia coli* secB gene product and demonstration of its activity in an in vitro protein translocation system. *J. Biol. Chem.* **264**, 2242-2249.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacterio-

- phage T4. *Nature* **227**, 680-685.
- Lamiet, A. A. and Plückthun, A. (1989). The precursor of β -lactamase: purification, properties and folding kinetics. *EMBO J.* **8**, 1469-1477.
- Lee, C., Li, P., Inouye, H., Brickman, E. R. and Beckwith, J. (1989). Genetic studies on the inability of β -galactosidase to be translocated across the *Escherichia coli* cytoplasmic membrane. *J. Bacteriol.* **171**, 4609-4616.
- Lill, R., Cunningham, K., Brundage, L. A., Ito, K., Oliver, D. and Wickner, W. (1989). SecA protein hydrolyzes ATP and is an essential component of the protein translocation ATPase of *Escherichia coli*. *EMBO J.* **8**, 961-966.
- Lill, R., Dowhan, W. and Wickner, W. (1990). The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. *Cell* **60**, 271-280.
- Lipinska, B., Fayet, O., Baird, L. and Georgopoulos, C. (1989). Identification, characterization, and mapping of the *Escherichia coli* *htrA* gene, whose product is essential for bacterial growth only at elevated temperatures. *J. Bacteriol.* **171**, 1574-1584.
- Lipinska, B., Zylicz, M. and Georgopoulos, C. (1990). The HtrA (DegP) protein, essential for *Escherichia coli* survival at high temperatures, is an endopeptidase. *J. Bacteriol.* **172**, 1791-1797.
- Lopilato, J., Bortner, S. and Beckwith, J. (1986). Mutations in a new chromosomal gene of *Escherichia coli* K-12, *pcnB*, reduce plasmid copy number of pBR322 and its derivatives. *Mol. Gen. Genet.* **205**, 285-290.
- MacIntyre, S., Mutschler, B. and Henning, U. (1991). Requirement of the SecB chaperone for export of a non-secretory polypeptide in *Escherichia coli*. *Mol. Gen. Genet.* **227**, 224-228.
- Matsuyama, S.-i., Fujita, Y. and Mizushima, S. (1993). SecD is involved in the release of translocated secretory proteins from the cytoplasmic membrane of *Escherichia coli*. *EMBO J.* **12**, 265-270.
- McCallum, C. D. and Epand, R. M. (1995). Insulin receptor autophosphorylation and signaling is altered by modulation of membrane physical properties. *Biochemistry* **34**, 1815-1824.
- McEwen, J. and Silverman, P. (1980a). Chromosomal mutations of *Escherichia coli* that alter expression of conjugative plasmid functions. *Proc. Natl. Acad. Sci. USA* **77**, 513-517.

- McEwen, J. and Silverman, P. (1980b). Mutations in genes *cpxA* and *cpxB* of *Escherichia coli* K-12 cause a defect in isoleucine and valine syntheses. *J. Bacteriol.* **144**, 68-73.
- McEwen, J. and Silverman, P. M. (1982). Mutations in genes *cpxA* and *cpxB* alter the protein composition of *Escherichia coli* inner and outer membranes. *J. Bacteriol.* **151**, 1553-1559.
- Mecasas, J., Rouviere, P. E., Erickson, J. W., Donohue, T. J. and Gross, C. A. (1993). The activity of σ^E , an *Escherichia coli* heat-inducible σ -factor, is modulated by expression of outer membrane proteins. *Genes & Dev.* **7**, 2618-2628.
- Mileykovskaya, E. and Dowhan, W. (1997). The Cpx two-component signal transduction pathway is activated in *Escherichia coli* mutant strains lacking phosphatidylethanolamine. *J. Bacteriol.* **179**, 1029-1034.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- Missiakas, D. and Raina, S. (1997). Signal transduction pathways in response to protein misfolding in the extracytoplasmic compartments of *E. coli*: role of two new phosphoprotein phosphatases PrpA and PrpB. *EMBO J.* **16**, 1670-1685.
- Mizuno, T., Chou, M.-Y. and Inouye, M. (1983). A comparative study on the genes for three porins of the *Escherichia coli* outer membrane. *J. Biol. Chem.* **258**, 6932-6940.
- Morona, R., Klose, M. and Henning, U. (1984). *Escherichia coli* K-12 outer membrane protein (OmpA) as a bacteriophage receptor: analysis of mutant genes expressing altered proteins. *J. Bacteriol.* **159**, 570-578.
- Nagasawa, H., Sakagami, Y., Suzuki, A., Suzuki, H., Hara, H. and Hirota, Y. (1989). Determination of the cleavage site involved in C-terminal processing of penicillin-binding protein 3 of *Escherichia coli*. *J. Bacteriol.* **171**, 5890-5893.
- Nakamura, M., Maruyama, I. N., Soma, M., Kato, J., Suzuki, H. and Hirota, Y. (1983). On the process of cellular division in *Escherichia coli*: nucleotide sequence of the gene for penicillin-binding protein 3. *Mol. Gen. Genet.* **191**, 1-9.
- Neidhardt, F. C. and Umbarger, H. E. (1996). Chemical composition of *Escherichia coli*. In *Escherichia coli and Salmonella: Cellular and Molecular Biology, Vol. 1, Second Edition* (ed. F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W.

- S. Reznikoff, M. Riley, M. Schaechter and H. E. Umbarger), pp. 13-16. Washington, D.C.: American Society for Microbiology.
- Neidhardt, F. C. and VanBogelen, R. A. (1987). Heat shock response. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (ed. F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter and H. E. Umbarger), pp. 1334-1345. Washington, D.C.: American Society for Microbiology.
- Neuhaus, J.-M., Schindler, H. and Rosenbusch, J. P. (1983). The periplasmic maltose-binding protein modifies the channel-forming characteristics of maltoporin. *EMBO J.* **2**, 1987-1991.
- Nikaido, H. and Reid, J. (1990). Biogenesis of prokaryotic pores. *Experientia* **46**, 174-180.
- Nishiyama, K.-i., Mizushima, S. and Tokuda, H. (1993). A novel membrane protein involved in protein translocation across the cytoplasmic membrane of *Escherichia coli*. *EMBO J.* **12**, 3409-3415.
- Oliver, D. B. (1993). SecA protein: autoregulated ATPase catalysing preprotein insertion and translocation across the *Escherichia coli* inner membrane. *Mol. Microbiol.* **7**, 159-165.
- Oliver, D. B. and Beckwith, J. (1982). Regulation of a membrane component required for protein secretion in *Escherichia coli*. *Cell* **30**, 311-319.
- Osborn, M. J., Gander, J. E., Parisi, E. and Carson, J. (1972). Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. *J. Biol. Chem.* **247**, 3962-3972.
- Park, S., Liu, G., Topping, T. B., Cover, W. H. and Randall, L. L. (1988). Modulation of folding pathways of exported proteins by the leader sequence. *Science* **239**, 1033-1035.
- Parkinson, J. S. and Kofoid, E. C. (1992). Communication modules in bacterial signaling proteins. *Annu. Rev. Genet.* **26**, 71-112.
- Pertinhez, T. A., Nakaie, C. R., Carvalho, R. S. H., Paiva, A. C. M., Tabak, M., Toma, F. and Schreier, S. (1995). Conformational changes upon binding of a receptor loop to lipid structures: possible role in signal transduction. *FEBS Lett.* **375**, 239-242.
- Pogliano, J. A. and Beckwith, J. (1994). SecD and SecF facilitate protein export in *Escherichia coli*. *EMBO J.* **13**, 554-561.
- Pogliano, J., Lynch, A. S., Belin, D., Lin, E. C. and Beckwith, J. (1997). Regulation of *Escherichia coli* cell envelope proteins involved in protein folding and degradation by the Cpx two-component system. *Genes Dev.* **11**, 1169-1182.

- Pugsley, A. P. (1993). The complete general secretory pathway in gram-negative bacteria. *Microbiol. Rev.* **57**, 50-108.
- Raina, S., Missiakas, D. and Georgopoulos, C. (1995). The *rpoE* gene encoding the σ^E (σ^{24}) heat shock sigma factor of *Escherichia coli*. *EMBO J.* **14**, 1043-1055.
- Raivio, T. L. and Silhavy, T. J. (1997). Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. *J. Bacteriol.* **179**, 7724-7733.
- Randall, L. L., Hardy, S. J. S. and Thom, J. R. (1987). Export of proteins: a biochemical view. *Annu. Rev. Microbiol.* **41**, 507-541.
- Richarme, G. (1982). Associative properties of the *Escherichia coli* galactose binding protein and maltose binding protein. *Biochem. Biophys. Res. Commun.* **105**, 476-481.
- Ried, G., Koebnik, R., Hindennach, I., Mutschler, B. and Henning, U. (1994). Membrane topology and assembly of the outer membrane protein OmpA of *Escherichia coli* K12. *Mol. Gen. Genet.* **243**, 127-135.
- Ronson, C. W., Nixon, B. T. and Ausubel, F. M. (1987). Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. *Cell* **49**, 579-581.
- Rouvière, P. E., De Las Peñas, A., Mecsas, J., Lu, C. Z., Rudd, K. E. and Gross, C. A. (1995). *rpoE*, the gene encoding the second heat-shock sigma factor, σ^E , in *Escherichia coli*. *EMBO J.* **14**, 1032-1042.
- Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. and Arnheim, N. (1985). Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**, 1350-1354.
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.
- Schwartz, M. (1987). The maltose regulon. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (ed. F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter and H. E. Umbarger), pp. 1482-1502. Washington, D.C.: American Society for Microbiology.
- Schweizer, M. and Henning, U. (1977). Action of a major outer cell envelope membrane protein in conjugation of *Escherichia coli*-12. *J. Bacteriol.* **129**, 1651-1652.

- Sen, K. and Nikaido, H. (1991). Trimerization of an in vitro synthesized OmpF porin of the *Escherichia coli* outer membrane. *J. Biol. Chem.* 266, 11295-11360.
- Silhavy, T. J., Shuman, H. A., Beckwith, J. and Schwartz, M. (1977). Use of gene fusions to study outer membrane protein localization in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 74, 5411-5415.
- Silverman, P. M. (1982). Gene *cpxA* is a new addition to the linkage map of *Escherichia coli* K-12. *J. Bacteriol.* 150, 425-428.
- Skórko-Glonek, J., Lipinska, B., Krzewski, K., Zolse, G., Bertoli, E. and Tanfani, F. (1997). HtrA heat shock protease interacts with phospholipid membranes and undergoes conformational changes. *J. Biol. Chem.* 272, 8974-8982.
- Snyder, W. B., Davis, L. J. B., Danese, P. N., Cosma, C. L. and Silhavy, T. J. (1995). Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway. *J. Bacteriol.* 177, 4216-4223.
- Snyder, W. B. and Silhavy, T. J. (1995). β -galactosidase is inactivated by intermolecular disulfide bonds and is toxic when secreted to the periplasm of *Escherichia coli*. *J. Bacteriol.* 177, 953-963.
- Sonntag, I., Schwarz, H., Hirota, Y. and Henning, U. (1978). Cell envelope and shape of *Escherichia coli*: Multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. *J. Bacteriol.* 136, 280-285.
- Spratt, B. G. (1977). Temperature-sensitive cell division mutants of *Escherichia coli* with thermolabile penicillin-binding proteins. *J. Bacteriol.* 131, 293-305.
- Stock, J. B., Ninfa, A. J. and Stock, A. M. (1989). Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol. Rev.* 53, 450-490.
- Strauch, K. L. and Beckwith, J. (1988). An *Escherichia coli* mutation preventing degradation of abnormal periplasmic proteins. *Proc. Natl. Acad. Sci. USA* 85, 1576-1580.
- Strauch, K. L., Johnson, K. and Beckwith, J. (1989). Characterization of *degP*, a gene required for proteolysis in the cell envelope and essential for growth of *Escherichia coli* at high temperature. *J. Bacteriol.* 171, 2689-2696.
- Stueber, D., Ibrahimi, I., Cutler, D., Dobberstein, B. and Bujard, H. (1984). A novel *in vitro* transcription-translation system: accurate and efficient synthesis of single proteins from cloned DNA sequences. *EMBO J.* 3, 3143-3148.

- Sugawara, E. and Nikaido, H. (1992). Pore-forming activity of OmpA protein of *Escherichia coli*. *J. Biol. Chem.* **267**, 2507-2511.
- Thome, B. M. and Müller, M. (1991). Skp is a periplasmic *Escherichia coli* protein requiring SecA and SecY for export. *Mol. Microbiol.* **5**, 2815-2821.
- Tommassen, J. and de Cock, H. (1995). Export and assembly of outer membrane proteins in *E. coli*. In *Advances in Cell and Molecular Biology of Membranes and Organelles*, Vol. 4 (ed. A. M. Tartakoff and R. E. Dalbey), pp. 145-173. Greenwich/CT: JAI Press.
- Van Alphen, L., Havekes, L. and Lugtenberg, B. (1977). Major outer membrane protein of *Escherichia coli* K-12. Purification and *in vitro* activity of bacteriophage K3 and F-pilus mediated conjugation. *FEBS Lett.* **75**, 285-290.
- Van Wielink, J. E. and Duine, J. A. (1990). How big is the periplasmic space? *Trends Biochem.* **15**, 136-137.
- Vidal-Ingigliardi, D. and Raibaud, O. (1991). Three adjacent binding sites for cAMP receptor protein are involved in the activation of the divergent *malEp-malKp* promoters. *Proc. Natl. Acad. Sci. USA* **88**, 229-233.
- Vögel, H. and Jähnig, F. (1986). Models for the structure of outer-membrane proteins of *Escherichia coli* derived from raman spectroscopy and prediction methods. *J. Mol. Biol.* **190**, 191-199.
- Wandersman, C., Schwartz, M. and Ferenci, T. (1979). *Escherichia coli* mutants impaired in maltodextrin transport. *J. Bacteriol.* **140**, 1-13.
- Wang, Q. and Kaguni, J. M. (1989). A novel sigma factor is involved in expression of the *rpoH* gene of *Escherichia coli*. *J. Bacteriol.* **171**, 4248-4253.
- Weber, R. F. and Silverman, P. M. (1988). The Cpx proteins of *Escherichia coli* K12: structure of the CpxA polypeptide as an inner membrane component. *J. Mol. Biol.* **203**, 467-478.
- Weiss, J. B., Ray, P. H. and Bassford, P. J. (1988). Purified SecB protein of *Escherichia coli* retards folding and promotes membrane translocation of the maltose-binding protein *in vitro*. *Proc. Natl. Acad. Sci. USA* **85**, 8978-8982.
- Wülfing, C. and Plückthun, A. (1994). Protein folding in the periplasm of *Escherichia coli*. *Mol. Microbiol.* **12**, 685-692.
- Yanisch-Perron, C., Vieira, J. and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.